## REMARKS

Claims 1-5, 7, 9, 11 and 15, 16 and 18 are pending in this application. Claims 1-3 and 5 remain pending but are withdrawn from examination as drawn to a non-elected invention.

Applicant has cancelled claims 6, 8, 10, 12-14, 17 and 19 without prejudice and without waiver of their right to file for and obtain claims directed to any subject matter in divisional and continuing applications which claim priority from this application.

Applicant has amended claims 4, 7, 9, 11, 15, 16 and 18 to promote clarity and to further define the scope of the invention.

Applicant has amended claims 4, 16 and 18 to specify that the sequences are "at least 75% identical to SEQ ID NO:...". Support for this amendment may be found on page 11, in Table 2; page 13, in Table 3; page 10, lines 16-19 and page 11, line 46-page 12, line 15 of the specification.

Applicant has amended claims 7 and 15 to delete their reference to cancelled claim 17.

None of the amendments adds new matter.

Applicant addresses the Examiner's rejections below:

**35 U.S.C. § 112, 2nd paragraph**

*Claims 4, 11, 16 and 17*

The Examiner has maintained the rejection of claims 4, 11, 16 and 17 under 35 U.S.C. § 112, second paragraph, as indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Specifically, the Examiner asserts that claims 4, 11 and 16 are indefinite in reciting "homologous sequences having at least 75% homology to SEQ ID NO:..." for reasons of record in rejecting the claims in the Office Action mailed December 15, 2000. In particular, the Examiner contends that the specification does not define either "homologous sequences" or "homology" in such a way that one of skill in the art could reasonably determine what applicant intends by the cited terminology.

Applicant has amended claims 4, 11 and 16 to delete the recitation "homologous sequences" or "homology" from the claims and substituted with the recitation that the sequences are "at least 75% identical to SEQ ID

NO:...". Support for this amendment may be found on page 11, in Table 2; page 13, in Table 3; page 10, lines 16-19 and page 11, line 46-page 12, line 15 of the specification. The rejection of claim 17 has been rendered moot by its cancellation herein. Accordingly, applicant requests that the Examiner withdraw this rejection.

**35 U.S.C. § 112, 1st paragraph**

*Claims 4, 7, 9, 11 and 15-18*

The Examiner has maintained the rejection of claims 4, 7, 9, 11 and 15-18 under 35 U.S.C. § 112, first paragraph, for allegedly lacking enablement. The Examiner contends that the specification does not enable a protein having at least 75% homology to SEQ ID NO: 4, or an antigenic or antibody-binding portion of SEQ ID NO: 4, nor an antigenic or antibody-binding portion of a protein having at least 75% homology to SEQ ID NO: 4, nor a viral subunit. The Examiner asserts that it would require undue experimentation for one of skill in the art to practice the invention as claimed since it encompasses proteins and fragments that are antigenic in any setting and that bind to any antibody. Applicant traverses, in part, based on the amendments and following remarks. Furthermore, the

rejection of claim 17 has been rendered moot by its cancellation herein.

To the extent that the Examiner views the specification as enabling for a protein having SEQ ID NO: 4, applicant agrees and states that, at a minimum, amended claims 4, 7, 9, 11, 15, 16 and 18 are enabled.

Applicants have amended claim 4 to recite a protein comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 4 or an amino acid sequence at least 75% identical to the amino acid sequence of SEQ ID NO:4, and antigenic fragments thereof. Applicants have amended claim 7 to recite a diagnostic kit comprising a protein according to claim 4. Applicants have amended claim 11 to recite a pharmaceutical composition comprising a protein according to claim 4. Applicants have amended claim 15 to recite a vaccine comprising a protein according to claim 4. Applicants have amended claim 16 to recite a method for preventing or treating infection in a mammal, including a human, by a virus comprising in the non-coding region of its viral genome a nucleotide sequence selected from the group consisting of SEQ ID NO:1 and sequences at least 75% identical to SEQ ID NO:1, said method comprising administering to said mammal a prophylactically or

therapeutically effective amount of a composition selected from the group consisting of: (a) a protein according to claim 4; (b) a pharmaceutical composition according to claim 11; and (c) a vaccine according to any one of claims 9, 15 or 18. Applicants have amended claim 18 to recite the vaccine according to claim 15, said vaccine further comprising a subunit of a virus, said virus comprising in the non-coding region of its viral genome a nucleotide sequence selected from the group consisting of SEQ ID NO:1 and sequences at least 75% identical to SEQ ID NO:1. Finally, applicants have amended claim 9 to recite the vaccine according to claim 15 or 18, further comprising an adjuvant.

The instant application teaches for the first time the discovery of new members of the picornavirus family known as Ljungan viruses and discloses both partial nucleotide and protein sequences of three Ljungan virus isolates (87-012, 174F and 145SL). The instant specification also describes that once the nucleotide and protein sequences of the three Ljungan virus isolates were taught, one of ordinary skill in the art would know how to determine whether a sequence is at least 75% identical with the Ljungan virus reference sequences using routine skills.

For example, once the polyprotein sequence of Ljungan virus isolate 145SL (SEQ ID NO: 4) was disclosed in the instant specification, one of ordinary skill in the art would be able to determine whether a protein is at least 75% identical to SEQ ID NO: 4 using routine skills by comparing and determining whether a sequence is at least 75% identical to SEQ ID NO: 4.

Furthermore, once the protein sequence of Ljungan virus isolate 145SL (SEQ ID NO: 4) was described in the instant specification, one of ordinary skill in the art could also readily determine, without any undue experimentation, whether a protein was an antigenic fragment of SEQ ID NO: 4, or an antigenic fragment of a protein at least 75% identical to SEQ ID NO: 4. As discussed in applicant's previous response dated January 29, 2003, an antigenic fragment refers to a protein fragment which can elicit an immune response. Such immune responses may include, but are not limited to, the production of immunizing or neutralizing antibodies.

Based on the teachings of the instant application, a person of skill in the art at the time of filing, having in hand the polyprotein sequence of Ljungan virus isolate 145SL (SEQ ID NO: 4) described herein, would

immediately recognize whether a protein was an antigenic fragment of SEQ ID NO: 4, or an antigenic fragment of a protein at least 75% identical to SEQ ID NO: 4 without any undue experimentation. *See* Janeway and Travers, *Immunobiology*, Third Edition, Chapter 2: The Induction, Measurement and Manipulation of the Immune Response, pp. 1-52 (1997), attached hereto as Exhibit A.

*Janeway and Travers* describes that antibodies bind stably and specifically to antigen via the antigen-binding site, which is the surface of the antibody molecule that makes physical contact with the antigen. The bound antibody can be then be visualized using a variety of sensitive techniques. *Id.* at page 2:20 and Glossary. In this instance, upon binding of an antibody to a protein having SEQ ID NO: 4 or a protein at least 75% identical to SEQ ID NO: 4 as described herein, one of ordinary skill in the art would be able to recognize whether a protein, which is capable of binding to the antibody, possesses an antigenic fragment of a protein having SEQ ID NO: 4 or a protein at least 75% identical to SEQ ID NO: 4. For at least the reasons stated, amended claims 4, 7, 9, 11, 15, 16 and 18 are fully enabled and applicant respectfully requests withdrawal of this rejection.

*Claims 15, 18, 9, 11, 16 and 19*

The Examiner has maintained the rejection of claims 15, 18, 9, 11, 16 and 19 under 35 U.S.C. § 112, first paragraph, for allegedly lacking enablement. Specifically, the Examiner asserts that applicant's previous arguments only show statistical correlation between the Ljungan virus isolates and human sera containing antibodies that immunoreact with the cells of the virus and that correlation is not equivalent to causation of any human disease. Furthermore, the Examiner asserts that even if it were to be discovered that the isolated viruses cause some human disease, the specification as filed does not teach how to use the parts of the virus disclosed as a vaccine to prevent or treat any disease. Applicant traverses.

First, applicant believes that the Examiner is mistaken in stating that claim 19 is rejected under 35 U.S.C. § 112, first paragraph because claim 19 was cancelled in applicant's previous response dated January 29, 2003. Furthermore, the Examiner has acknowledged the cancellation of claim 19 in the instant Office Action dated April 10, 2003.

Second, the MPEP states that the issue of "correlation" as raised by the Examiner refers to the relationship between *in vitro* or *in vivo* animal model assays and a disclosed or claimed method of use. The issue of "correlation" is also dependent on the state of the art such that if the one in the art recognizes a particular model as correlating to a particular condition, then it should be accepted as correlating. MPEP 2164.02. Furthermore, a rigorous or an invariant exact correlation is not required, as stated in *Cross* v. *Iizuka*, 753 F.2d 1040, 1050, 224 USPQ 739, 747 (Fed. Cir. 1985):

> [B]ased upon the relevant evidence as a whole, there is a reasonable correlation between the disclosed in vitro utility and an in vivo activity, and therefore a rigorous correlation is not necessary where the disclosure of pharmacological activity is reasonable based upon the probative evidence.

The instant specification discloses the isolation of three different Ljungan viral isolates which exhibit cytopathic effect (CPE) and react with a panel of human sera isolated from patients with multiple sclerosis, Diabetes Mellitus and myocarditis using immunofluorescence test (IFT). Furthermore, electron microscopy revealed that

the three Ljungan viral isolates displayed sizes and structures compatible with a picornavirus and alignment of their sequences further revealed substantial homology both at the nucleotide and amino acid levels with known cardiovirus sequences. Moreover, the three Ljungan viral isolates were able to kill suckling mice when administered intracerebrally into 1-day old suckling mice. Furthermore, the specification discloses that sera collected from patients with diabetes mellitus or myocarditis immunoreacted positively with cells infected with the three Ljungan viral isolates. Thus, based upon the relevant evidence as a whole, there is reasonable correlation between the disclosed *in vitro* utility and an *in vivo* activity.

Applicants have amended claims 15, 18 and 9 to recite a vaccine comprising a protein according to claim 4, said vaccine further comprising a subunit of a virus comprising in the non-coding region a nucleotide sequence selected from the group consisting of SEQ ID NO: 1 or sequences at least 75% identical to SEQ ID NO: 1, and further comprising an adjuvant, respectively. Amended claim 11 is directed to a pharmaceutical composition comprising a protein according to claim 4. Amended claim

16 is directed to a method for preventing or treating infection by a virus by administering to said mammal a composition from the group selected from: (a) a protein according to claim 4, (b) a pharmaceutical composition according to claim 11 or (c) a vaccine according to any one of claims 9, 15 or 18.

As discussed earlier, the instant application teaches for the first time the partial nucleotide and protein sequences of three Ljungan virus isolates such that one of ordinary skill in the art would know how to determine whether a sequence is at least 75% identical with the Ljungan virus reference sequences using routine skills. Furthermore, once the polyprotein sequence of Ljungan virus isolate 145SL (SEQ ID NO: 4) was described in the instant specification, one of ordinary skill in the art could also readily determine, based on its ability to bind to antibodies, whether the protein was an antigenic fragment of SEQ ID NO: 4, or an antigenic fragment of a protein is at least 75% identical to SEQ ID NO: 4.

Based on the teachings of the instant application, a person of skill in the art at the time of filing, having in hand the polyprotein sequence of Ljungan virus isolate 145SL (SEQ ID NO: 4) described herein, would

immediately recognize how to develop a vaccine comprising a protein according to claim 4 without undue experimentation based on the determination of whether the protein was an antigenic fragment of SEQ ID NO: 4, or an antigenic fragment of a protein at least 75% identical to SEQ ID NO: 4.

Furthermore, a person of ordinary skill in the art at the time of filing, having in hand the polyprotein sequence of Ljungan virus isolate 145SL (SEQ ID NO: 4) described herein, would recognize how to generate pharmaceutical compositions for treating or preventing infection by a virus comprising a protein according to claim 4 based on whether the protein was an antigenic portion of SEQ ID NO: 4, or an antigenic portion of a protein at least 75% identical to SEQ ID NO: 4 without undue experimentation.

Similarly, a person of skill in the art at the time of filing, having in hand the polyprotein sequence of Ljungan virus, would recognize a method for preventing or treating infection by a virus by administering to said mammal a composition from the group selected from a protein according to claim 4, a pharmaceutical composition

according to claim 11 or a vaccine according to any one of claims 9, 15 or 18 without undue experimentation.

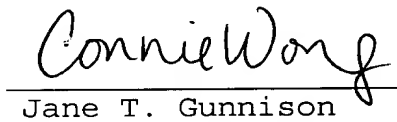
For at least the reasons stated, amended claims 15, 18, 9, 11 and 16 are fully enabled and the Examiner's rejection has been obviated.

## CONCLUSION

For all the above reasons, applicant requests that the Examiner withdraw all outstanding rejections and grant allowance of the pending claims.

The Examiner is invited to telephone applicant's representatives regarding any matter that may be handled by telephone to expedite allowance of the pending claims.

Respectfully submitted,



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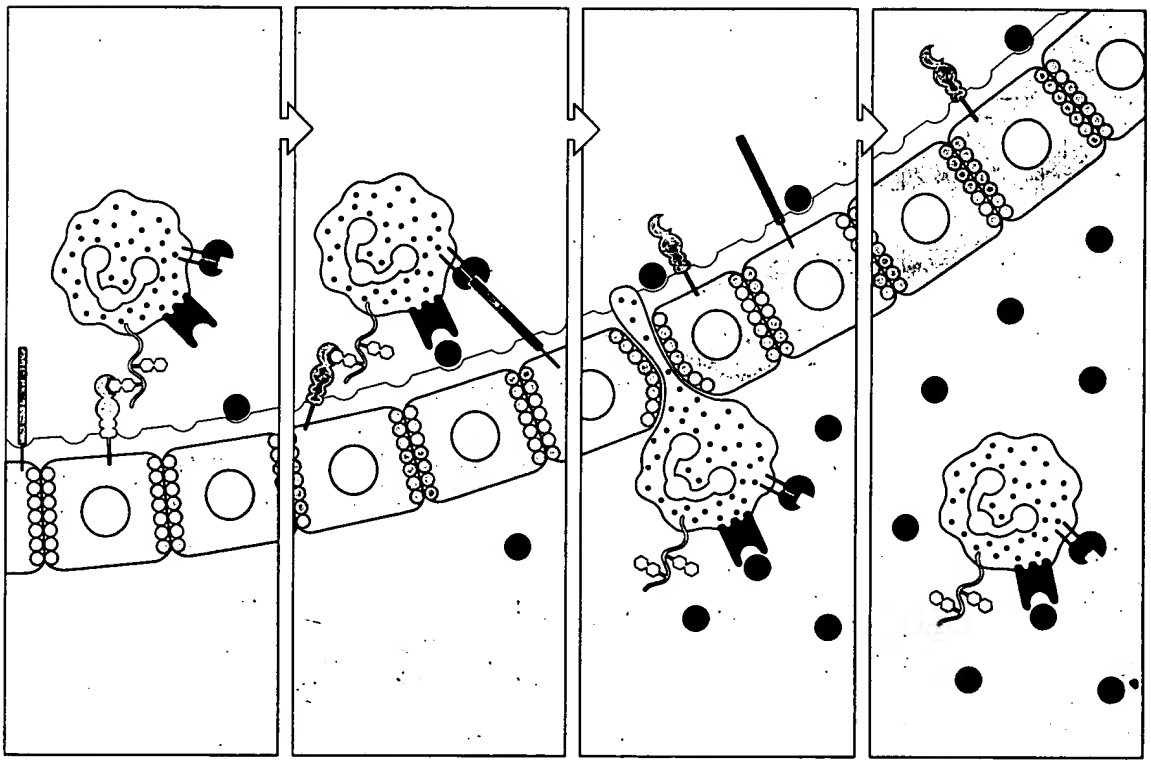
# IMMUNOBIOLOGY

## THE IMMUNE SYSTEM IN HEALTH AND DISEASE

## THIRD EDITION



## JANEWAY-TRAVERS

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# The Induction, Measurement, and Manipulation of the Immune Response

# 2


Before you read further, a word from the authors about this chapter. We have written it to be read when it is needed to understand a particular method; later chapters reference Chapter 2 as appropriate. Although it is also written so that it can be read from start to finish, most students will want to dip into this toolbox of methods when they encounter a reference to it in later chapters, rather than tackling it now. To make the sections relevant to later chapters easy to identify, the edges of the paper in this chapter are colored, and the most useful methods for any part of the book are color coded to the part in which they are needed. However, we recommend that you read the first six sections of Chapter 2 before continuing with the rest of the book.

The description of the immune system outlined in Chapter 1 is drawn from the results of many different kinds of experiment and from the study of human disease. Immunologists have devised a wide variety of techniques for inducing, measuring, and characterizing immune responses, and for altering the immune system through cellular, molecular, and genetic manipulation. Before we examine the cellular and molecular basis of host defense described in the remainder of this book, we shall look at how the immune system is studied and introduce the specialized language of immunology. In this chapter, we also describe many basic immunological phenomena that experimental immunologists seek to explain in terms of the cellular and molecular features of the immune system. Since genetics plays an important role in the analysis of the immune system and of human disease, the genetic analysis of the immune system is also discussed here, including recently developed techniques for genetic manipulation that have had a tremendous impact on all areas of biology. We also describe clinical tests used to assess immune function in patients with immunological disorders.

Immunological techniques are also widely applied in many other areas of biology and medicine. The use of antibodies to detect specific molecules in complex mixtures and in tissues is of particular importance. We therefore devote an entire section of this chapter to the antibody-based methods used by immunologists, by basic scientists in many other biological disciplines, and by clinicians. These methods illustrate the specificity and utility of antibodies, whose structure and generation form an important theme in subsequent parts of this book.

elated
› Part I

# The induction and detection of immune responses.

Most of the material in this book focuses on **adaptive immunity**, that is, on immune responses of lymphocytes to foreign materials, most importantly the antigens borne by various pathogenic microorganisms. However, experimental immunologists, in developing our understanding of the immune response, have mainly examined responses induced by simple non-living antigens. Thus, we shall begin our consideration of how the immune system is studied by discussing how such adaptive immune responses are induced and detected. The deliberate induction of an immune response is known as **immunization**. Experimental immunizations are carried out routinely by injecting the test antigen into the animal or human subject, and we shall see that the route, dose, and form in which antigen is administered can profoundly affect whether a response occurs and the type of response that is produced. To determine whether an immune response has occurred and to follow its course, the immunized individual is monitored for the appearance of immune reactants directed at the specific antigen. Immune responses to most antigens elicit the production of both specific antibodies and specific effector T cells. Monitoring the antibody response often involves analysis of relatively crude preparations of **antiserum** (plural: **antisera**). This is the fluid phase of clotted blood (the **serum**), which, in an immunized individual, is called antiserum because it contains specific antibodies against the immunizing antigen as well as other soluble serum proteins. To study immune responses mediated by T cells, blood lymphocytes or cells from lymphoid organs are tested; T-cell responses are more commonly studied in experimental animals than in humans.

Any substance that can elicit an immune response is said to be **immunogenic** and is called an **immunogen**. There is a clear operational distinction between an immunogen and an antigen. An antigen is defined as any substance that can bind to a specific antibody. All antigens therefore have the potential to elicit specific antibodies but some need to be attached to an immunogen in order to do so. This means that although all immunogens are antigens, not all antigens are immunogenic.

The following sections describe some of the most commonly used techniques for inducing, detecting, and measuring adaptive immune responses. These techniques are used to address many questions in immunology. What determines whether a particular substance will be immunogenic or not? How does one raise antibodies against substances that are not by themselves immunogenic? And what determines which type of response will be provoked by a particular immunization? We shall first examine the nature of antigens and the features that make a substance immunogenic, before turning to a general consideration of how the response is detected.

## 2-1 Antibodies can be produced against almost any substance.

When antibodies were first discovered as the agents of resistance to infection, it was thought likely that their ability to bind pathogens had been selected over evolutionary time because of their importance to survival. However, Karl Landsteiner soon showed that antibodies could be elicited against a virtually limitless range of molecules, including synthetic chemicals never found in the natural environment. This demonstrated unequivocally that the repertoire of possible antibodies in any

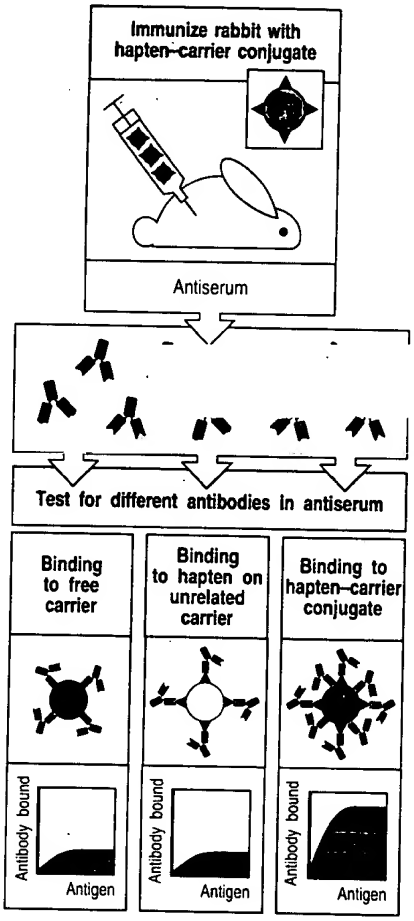



**Fig. 2.1 Antibodies can be elicited by small chemical gr ups called haptens only when the hapten is linked t a protein carrier.** Three types of antibodies are produced. One set (blue) binds the carrier protein alone and is called carrier-specific. One set (red) binds to the hapten on any carrier or to free hapten in solution and is called hapten-specific. One set (purple) only binds the specific conjugate of hapten and carrier used for immunization, apparently binding to sites at which the hapten joins the carrier, and is called conjugate-specific. The amount of antibody of each type in this serum is shown schematically in the graphs at the bottom; note that the original antigen binds more antibody than the sum of anti-hapten and anti-carrier owing to the additional binding of conjugate-specific antibody.

individual is essentially unlimited, and that the genes encoding individual antibodies could not have been selected for their action against pathogens. This radically changed the way that immunologists thought about the antibody response, forcing them to the conclusion that evolution must have selected not for specific antibody structures but rather for the ability to generate an open repertoire of antibodies of diverse structure, a subject we shall focus on in Chapter 3. This meant that no two individuals are likely to make the same response to any antigen. It also alerted immunologists to the potential utility of antibodies for detecting and measuring almost any substance in a complex mixture of molecules.

In order to determine the range of antibodies that could be produced, Landsteiner studied the immune response to small organic molecules, such as arsonates and nitrophenyls. Although these simple structures do not provoke antibodies when injected by themselves, Landsteiner found that antibodies could be raised against them if the molecule was attached covalently to a protein carrier. He therefore termed them **haptens** (from the Greek *haptein*, to fasten). Animals immunized with a hapten–carrier conjugate produced three distinct sets of antibodies (Fig. 2.1). One set comprised hapten-specific antibodies that reacted with the hapten on any carrier, as well as with free hapten. The second set of antibodies was specific for the carrier protein, as shown by their ability to bind both the hapten-modified and unmodified carrier protein. Finally, some antibodies reacted only with the specific conjugate of hapten and carrier used for immunization. Landsteiner studied mainly the antibody response to the hapten, as these small molecules could be synthesized in many closely related forms. As can be seen in Fig. 2.2, antibodies raised against a particular hapten bind that hapten but, in general, fail to bind even very closely related chemical structures. The binding of haptens by anti-hapten antibodies has played an important part in defining the precision of antigen binding by antibody molecules. Anti-hapten antibodies are also important medically as they mediate allergic reactions to penicillin and other compounds that elicit antibody responses when they attach to self proteins (see Section 11-10).

Antisera contain many different antibody molecules that bind to the immunogen in slightly different ways (see Fig. 2.1 and Fig. 2.2). Some of the antibodies in an antiserum are cross-reactive. A **cross-reaction** is defined as the binding of an antibody to an antigen other than the immunogen; most cross-react with closely related molecules but some are specific for antigens having no clear relationship to the immunogen.

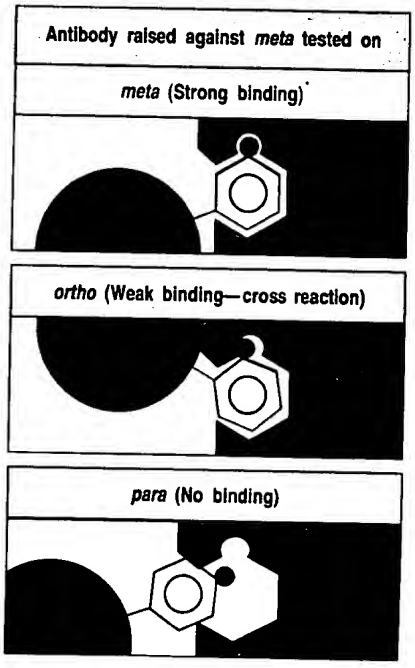



**Fig. 2.2 Anti-hapten antib dies can distinguish small changes in hapten structure.** Antibodies raised to the *meta* substituted azobenzenearsonate ring react predominantly with the *meta* form, and have limited or no cross-reactivity with the *ortho* and *para* forms. The particular antibody shown here fits the *meta* form perfectly, weakly binds to the *ortho* form, and does not bind the *para* form.

These cross-reacting antibodies can create problems when the antiserum is used for detection of specific antigen using the techniques outlined in the next part of this chapter. They can be removed from an antiserum by **absorption** with the cross-reactive antigen, leaving behind the antibodies that bind only to the immunogen. This can be performed using immobilized antigen by affinity chromatography, which is also used for purification of antibodies or antigens (see Section 2-7). The problems resulting from the heterogeneity of the antibodies present in an antiserum can be avoided by making monoclonal antibodies, which are homogeneous antibodies derived from a single antibody-producing cell (see Section 2-11). These can be selected for lack of cross-reactivity and their binding properties can be defined more reliably.

Related to Part I

The antigens used most frequently in experimental immunology are proteins, and antibodies to proteins are of enormous utility in experimental biology and medicine. Therefore, in this chapter we shall focus on the production and use of anti-protein antibodies. While antibodies can also be made to haptens, to carbohydrates, to nucleic acids, and to other structural classes of antigen (see Chapter 8), their induction generally requires the attachment of the antigen to a protein carrier. Thus, the immunogenicity of protein antigens determines the outcome of virtually every immune response.

## 2-2 The immunogenicity of a protein depends on its presentation to T cells.

Although any structure can be recognized by antibody as an antigen, usually only proteins elicit fully developed adaptive immune responses because of their ability to engage the T cells which contribute to inducing most antibody responses and which are required for immunological memory. Proteins engage T cells because the T cells recognize antigens

**Fig. 2.3 Intrinsic properties and extrinsic factors that affect the immunogenicity of proteins.**

**Factors that influence the immunogenicity of proteins**

| Parameter | Increased immunogenicity | Decreased immunogenicity |
|---|---|---|
| Size | Large | Small (MW<2 500) |
| Dose | Intermediate | High or low |
| Route | Subcutaneous > intraperitoneal > intravenous or intragastric | |
| Composition | Complex | Simple |
| Form | Particulate | Soluble |
| | Denatured | Native |
| Similarity to self protein | Multiple differences | Few differences |
| Adjuvants | Slow release | Rapid release |
| | Bacteria | No bacteria |
| Interaction with host MHC | Effective | Ineffective |

as peptide fragments of proteins bound to major histocompatibility complex (MHC) molecules (see Section 1-14). An adaptive immune response that includes immunological memory can only be induced by other classes of antigen when they are attached to a protein carrier that can engage the necessary T cells. Immunological memory is produced as a result of the initial or **primary immunization**. This is also known as **priming**, as the animal or person is now primed to mount a more potent response to subsequent challenges by the same antigen. The response to each challenge is increasingly intense, so that **secondary**, **tertiary**, and subsequent responses are of increasing magnitude. Repetitive injection of antigen to achieve a heightened state of immunity is known as **hyperimmunization**.

Certain properties of a protein that favor the priming of an adaptive immune response have been defined by studying antibody responses to simple natural proteins like hen egg-white lysozyme and, more importantly, to synthetic polypeptide antigens (Fig. 2.3). The larger and more complex a protein, and the more distant its relationship to self proteins, the more likely it is to elicit a response. This is because such responses depend on the protein being degraded into peptides that can bind to MHC molecules, and on the subsequent recognition of these peptide: MHC complexes by T cells that have survived a process of selection against responsiveness to self. Particulate or aggregated antigens are more immunogenic because they are taken up more efficiently by the specialized antigen-presenting cells responsible for initiating a response (see Section 1-11); indeed small soluble proteins are unable to induce a response unless they are made to aggregate in some way. As we shall see in Sections 2-3 and 2-4, the way in which protein antigens are administered can greatly influence the induction and character of the immune response. For the purposes of this chapter we are focusing on empirical observations relevant to the practice of immunization. A clearer understanding of how these various parameters determine immunogenicity will become evident when the priming of T cells is described in Chapter 7.

## 2-3 The response to a protein antigen is influenced by dose, form, and route of administration.

The magnitude of the immune response depends on the dose of immunogen administered. Below a certain threshold dose, most proteins do not elicit an immune response. Above the threshold dose, there is a gradual increase in the response with increasing dose to a broad plateau level, followed by a decline at very high antigen doses (Fig. 2.4). As most infectious agents enter the body in small numbers, immune responses are generally elicited only by pathogens that multiply to a level sufficient to exceed the antigen dose threshold. The broad-response optimum allows the system to respond to infectious agents across a wide range of doses. At very high antigen doses the immune response is inhibited, which may be important in maintaining tolerance to abundant self proteins, such as plasma proteins. In general, secondary and subsequent immune responses occur at lower antigen doses and achieve higher plateau values, which is a sign of immunological memory. However, under some conditions, very low or very high doses of antigen may induce specific unresponsive states, known respectively as acquired **low-zone** or **high-zone tolerance**.

The route by which antigen is administered also affects both the magnitude and the type of response obtained. Antigens injected subcutaneously generally elicit the strongest responses, while antigens injected or transfused directly into the bloodstream, especially those freed of aggregates

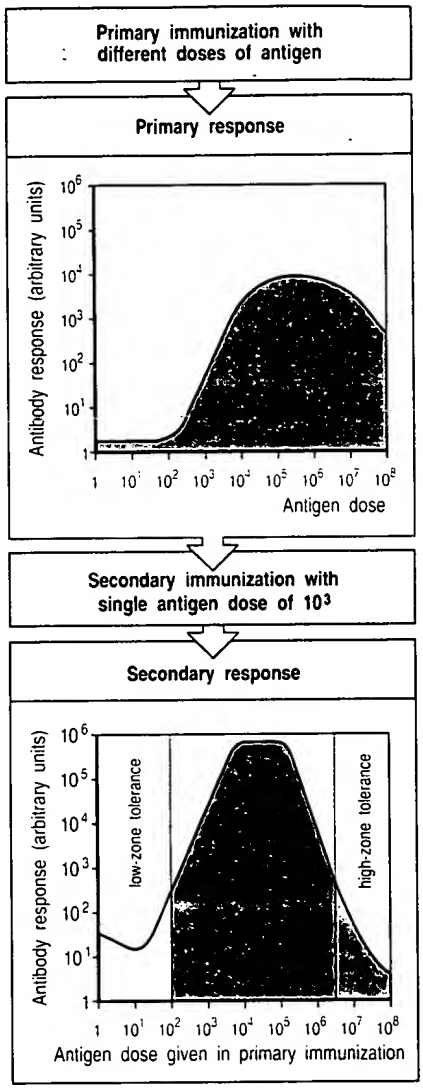



**Fig. 2.4 The dose of antigen used in an initial immunization affects the primary and secondary antibody response.** The typical antigen dose–response curve shown here illustrates the influence of dose on both a primary antibody response (amounts of antibody produced expressed in arbitrary units) and the effect of the dose used for priming on a secondary antibody response elicited by a dose of antigen of $10^3$ arbitrary mass units. Very low doses of antigen do not cause an immune response at all. Slightly higher doses appear to inhibit specific antibody production, an effect known as low-zone tolerance. Above these doses there is a steady increase in the response with antigen dose to reach a broad optimum. Very high doses of antigen also inhibit immune responsiveness to a subsequent challenge, a phenomenon known as high-zone tolerance.

Relate to Part

that are readily taken up by antigen-presenting cells, tend to induce unresponsiveness or tolerance unless they bind to host cells. Antigens administered solely to the gastrointestinal tract have distinctive effects, frequently eliciting a local antibody response in the intestinal lamina propria, while at the same time producing a state of systemic tolerance that manifests as a diminished response to the same antigen if it is administered in immunogenic form elsewhere in the body. This 'split tolerance' may be important in avoiding allergy to antigens in food, as the local response prevents food antigens from entering the body. The inhibition of systemic immunity helps to prevent formation of IgE antibodies, which are the cause of such allergies (see Chapter 11). By contrast, protein antigens that enter the body through the respiratory epithelium tend to elicit allergic responses, for reasons that are not clear.

Related to Part I

## 2-4 Immunogenicity can be enhanced by administration of proteins in adjuvants.

Most proteins are poorly immunogenic or non-immunogenic when administered by themselves. Strong adaptive immune responses to protein antigens almost always require that the antigen be injected in a mixture known as an **adjuvant**. An adjuvant is any substance that enhances the immunogenicity of substances mixed with it. Adjuvants differ from protein carriers in that they do not form stable linkages with the immunogen. Furthermore, adjuvants are needed primarily in initial immunizations, whereas carriers are required to elicit not only primary but also subsequent responses to haptens. Commonly used adjuvants are listed in Fig. 2.5.

Adjuvants can enhance immunogenicity in two different ways. First, adjuvants convert soluble protein antigens into particulate material,

**Fig. 2.5 Common adjuvants and their use.** Adjuvants are mixed with the antigen and usually render it particulate, which helps to retain the antigen in the body and promotes macrophage uptake. Most adjuvants include bacteria or bacterial components that stimulate macrophages, aiding in the induction of the immune response. ISCOMs (immune stimulatory complexes) are small micelles of the detergent Quil A; when viral proteins are placed in these micelles, they apparently fuse with the antigen-presenting cell, allowing the antigen to enter the cytosol and stimulate a response to the protein, much as a virus infecting these cells would stimulate an anti-viral response.

**Adjuvants that enhance immune responses**

| Adjuvant name | Composition | Mechanism of action |
|---|---|---|
| Incomplete Freund's adjuvant | Oil-in-water emulsion | Delayed release of antigen; enhanced uptake by macrophages |
| Complete Freund's adjuvant | Oil-in-water emulsion with dead mycobacteria | Delayed release of antigen; enhanced uptake by macrophages; induction of co-stimulators in macrophages |
| Freund's adjuvant with MDP | Oil-in-water emulsion with muramyldipeptide (MDP), a constituent of mycobacteria | Similar to complete Freund's adjuvant |
| Alum (aluminum hydroxide) | Aluminum hydroxide gel | Delayed release of antigen; enhanced macrophage uptake |
| Alum plus *Bordetella pertussis* | Aluminum hydroxide gel with killed *B. pertussis* | Delayed release of antigen; enhanced uptake by macrophages; induction of co-stimulators |
| Immune stimulatory complexes (ISCOMs) | Matrix of Quil A containing viral proteins | Delivers antigen to cytosol; allows induction of cytotoxic T cells |

which is more readily ingested by antigen-presenting cells, such as macrophages. The antigen can be adsorbed on particles of the adjuvant (such as alum) or made particulate by emulsification in mineral oils. This enhances immunogenicity somewhat, but such adjuvants are relatively weak unless they also contain bacteria or bacterial products, the second means by which adjuvants enhance immunogenicity. Although the exact contribution of the microbial constituents to enhancing immunogenicity is unknown, they are clearly the more important component of an adjuvant. Microbial products may signal macrophages or dendritic cells to become more effective antigen-presenting cells, and their role is considered in more detail in Chapter 7. The bacterial constituents in most adjuvants induce the production of inflammatory cytokines and potent local inflammatory responses; this effect is probably intrinsic to their activity in enhancing responses, but precludes their use in humans. Nevertheless, purified constituents of the bacterium *Bordetella pertussis*, which is the causal agent of whooping cough, are used as both antigen and adjuvant in the triplex DPT (diphtheria, pertussis, tetanus) vaccine against these diseases.

Relate to Part

## 2-5 B-cell responses are detected by antibody production.

B cells contribute to adaptive immunity by secreting antibodies, and the response of B cells to an injected immunogen is usually measured by analyzing the specific antibody produced in a **humoral immune response**. This is most conveniently achieved by assaying the antibody that accumulates in the fluid phase of the blood or **plasma**; such antibodies are known as circulating antibodies. Circulating antibody is usually measured by collecting blood, allowing it to clot, and then isolating the serum from the clotted blood. The amount and characteristics of the antibody in the resulting antiserum are then determined using the assays we shall describe in the next part of this chapter.

The most important characteristics of an antibody response are the specificity, amount, isotype (class), and affinity of the antibodies produced. The specificity determines the ability of the antibody to distinguish the immunogen from other antigens. The amount of antibody can be determined in many different ways and is a function of the number of responding B cells, their rate of antibody synthesis, and the persistence of the antibody after production. The persistence of an antibody in the plasma and extracellular fluid bathing the tissues is determined by its isotype (see Chapter 3); each isotype has a different half-life *in vivo*. The isotypic composition of an antibody response also determines the biological functions these antibodies can perform and the sites in which antibody will be found. Finally, the strength of binding of the antibody to its antigen is termed its **affinity**. Binding strength is important, since the higher the affinity of the antibody for its antigen, the less antibody is required to eliminate the antigen, as antibodies with higher affinity will bind at lower antigen concentrations. All these parameters of the humoral immune response help to determine the capacity of that response to protect the host from infection.

## 2-6 T-cell responses are detected by their effects on other cells or by the cytokines they produce.

The measurement of antibody responses in humoral immunity is fairly simple; by contrast, immunity that is mediated by T cells, called **cell-mediated immunity**, is technically far more difficult to measure. This is principally because T cells do not make a secreted antigen-binding

product, so there is no simple binding assay for their antigen-specific responses. T-cell activity can be divided into an induction phase, in which T cells are activated to divide and differentiate, and an effector phase, in which their function is expressed. Both phases require an interaction between two cells, in which the T cell recognizes specific antigen displayed in the form of peptide:MHC complexes on the surface of the interacting cell. In the induction phase, the interaction must be with an antigen-presenting cell able to deliver co-stimulatory signals, whereas, in the effector phase, the appropriate target cell depends on the type of armed effector T cell that has been activated. Most commonly, the presence of T cells that have responded to a specific antigen is detected by their subsequent *in vitro* proliferation when re-exposed to the same antigen. However, T-cell proliferation only indicates that cells able to recognize that antigen have been activated previously; it does not reveal what effector function they mediate. The effector function of a T cell is assayed by its effect on an appropriate target cell. As we learned in Chapter 1, several basic effector functions have been defined for T cells. Cytotoxic CD8 T cells can kill infected target cells, thus preventing further replication of obligate intracellular pathogens, while CD4 T cells can activate either B cells or macrophages in ways that are determined largely by the cytokines they produce (see Section 7-17). The different T-cell effector responses that can be elicited by immunization determine the functional outcome of an immune response. However, no general principles that allow one to predict the type of immune response produced by a particular immunization regimen have emerged. The ability to control the type of immune response produced remains a central goal of immunology as we shall see in Chapters 9–13.

Related to Part II

**Summary.**

Adaptive immunity is studied by eliciting a response through deliberate infection or, more commonly, by injection of antigens in an immunogenic form, and by measuring the outcome in terms of humoral and cell-mediated immunity. Intrinsic properties of the antigen determine its immunogenic potential. However, the elicitation of an immune response is heavily influenced by the dose and route of antigen administration and by the adjuvants used to administer it. The main parameters of the antibody response are the amount, affinity, isotype, and specificity of the antibody produced, the isotype determining the functional capabilities of the humoral immune response to a given antigen. The main parameters of the cell-mediated immune response are the numbers of T cells able to respond and their functional properties.

## The measurement and use of antibodies.

Antibody molecules are highly specific for their corresponding antigen, being able to detect one molecule of a protein antigen out of more than $10^8$ similar molecules. This makes antibodies both easy to isolate and study, and invaluable as probes of biological processes. While standard chemistry would have great difficulty in distinguishing two such closely related proteins as human and pig insulin, antibodies can be made that discriminate between these two structures absolutely. The utility of antibodies as molecular probes has stimulated the development of many sensitive and highly specific techniques to measure their presence, to

determine their specificity and affinity for a range of antigens, and to ascertain their functional capabilities. Many standard techniques used throughout biology exploit the specificity and stability of antigen binding by antibodies. Comprehensive guides to the conduct of these antibody assays are available in many books on immunological methodology; we shall illustrate here only the most important techniques, especially those used in studying the immune response itself. These examples also illustrate the unique properties of antibody molecules that are explained by their structure and genetic origin, as we shall see in Chapter 3.

## 2-7 The amount and specificity of an antibody can be measured by its direct binding to antigen.

The presence of specific antibody can be detected using many different assays. Some measure the direct binding of the antibody to its antigen. Such assays are based on **primary interactions** and we shall describe several in this section. Others determine the amount of antibody present by the changes it induces in the physical state of the antigen, such as the precipitation of soluble antigen or the clumping of antigenic particles; these are called **secondary interactions** and will be described in the next section. Both types of assay can be used to measure the amount and specificity of the antibodies produced after immunization, and both can be applied to a wide range of other biological problems. Here, we shall describe several of these assays that are commonly used in immunology, biology, and medicine. As such assays were originally conducted using sera from immune individuals, or antisera, they are commonly referred to as **serological assays**, and the use of antibodies is often called **serology**. The amount of antibody is usually determined by titration of the antiserum by serial dilution, and the point at which binding falls to 50% of the maximum is usually referred to as the **titer** of an antiserum.

Two commonly used direct binding assays are **radioimmunoassay (RIA)** and **enzyme-linked immunosorbent assay (ELISA)**. For these one needs a pure preparation of a known antigen or antibody, or both. In a radioimmunoassay, a pure component (antigen or antibody) is radioactively labeled, usually with $^{125}I$. For the ELISA, an enzyme is linked chemically to the antibody or antigen. The unlabeled component (again either antigen or antibody) is attached to a solid support, such as the wells of a plastic multiwell plate, which will adsorb a certain amount of any protein. Most commonly, the antigen is attached to the solid support and the binding of labeled antibody is assayed. The labeled antibody is allowed to bind to the unlabeled antigen, under conditions where non-specific adsorption is blocked, and any unbound antibody and other proteins are washed away. Antibody binding is measured directly in terms of the amount of radioactivity retained by the coated wells in radioimmunoassay, while in ELISA, binding is detected by a reaction that converts a colorless substrate into a colored reaction product (Fig. 2.6). The color change can be read directly in the reaction tray, making data collection very easy, and ELISA also avoids the hazards of radioactivity. This makes ELISA the preferred method for most direct-binding assays.

These assays illustrate two crucial aspects of all serological assays. First, at least one of the reagents must be available in a pure, detectable form in order to obtain quantitative information. Second, there must be a means of separating the bound fraction of the labeled reagent from the unbound, free fraction so that specific binding can be determined. Normally, this separation is achieved by having the unlabeled partner

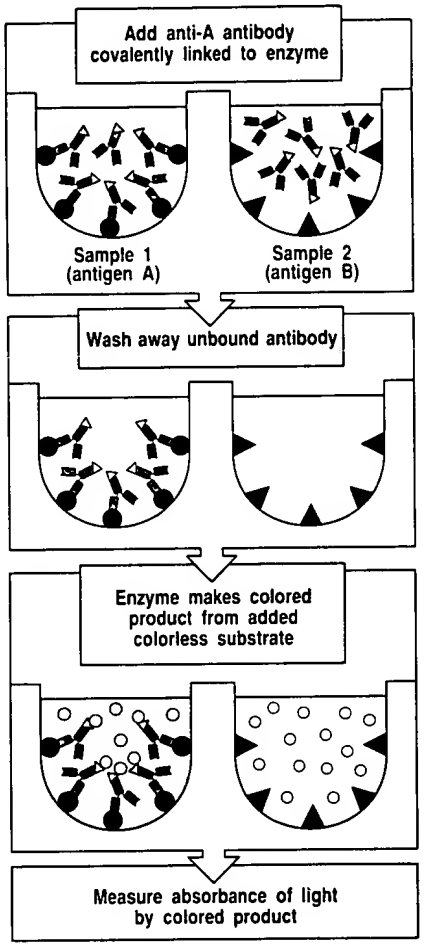



**Fig. 2.6 The principle of the enzyme-linked immunosorbent assay (ELISA).** To detect antigen A, purified antibody specific for antigen A is linked chemically to an enzyme. The samples to be tested are coated onto the surface of plastic wells to which they bind non-specifically; residual sticky sites on the plastic are blocked by adding irrelevant proteins (not shown). The labeled antibody is then added to the wells under conditions where non-specific binding is prevented, so that only binding to antigen A causes the labeled antibody to be retained on the surface. Unbound labeled antibody is removed from all wells by washing, and bound antibody is detected by an enzyme-dependent color-change reaction. This assay allows arrays of wells known as microtiter plates to be read in fiberoptic multichannel spectrometers, greatly speeding the assay. Modifications of this basic assay allow antibody or antigen in unknown samples to be measured as shown in Figs. 2.7 and 2.34 (see also Section 2-9).

trapped on a solid support, allowing the labeled partner that binds to be separated from the unbound labeled molecules by washing. In Fig. 2.6, the unlabeled antigen is attached to the well and the labeled antibody is trapped by binding to it. This separation of bound from free is an essential step in every assay that uses antibodies.

These assays do not allow one to measure directly the amount of antigen or antibody in a sample of unknown composition, as both depend on binding of a pure labeled antigen or antibody. There are various ways around this problem, one of which is to use a competitive inhibition assay, as shown in Fig. 2.7. In this type of assay, the presence and amount of a particular antigen in an unknown sample is determined by its ability to compete with a labeled reference antigen for binding to an antibody attached to a plastic well. By adding varying amounts of a known, unlabeled standard preparation, a standard curve is constructed, and the assay can then measure the amount of antigen in unknown samples by comparison to the standard. The competitive binding assay can also be used for measuring antibody in a sample of unknown composition by attaching the appropriate antigen to the plate and measuring the ability of the test sample to inhibit the binding of a labeled specific antibody.

Related to Part II

All the assays described so far rely on pure preparations of an antibody. However, antibody to any one antigen makes up a very small percentage

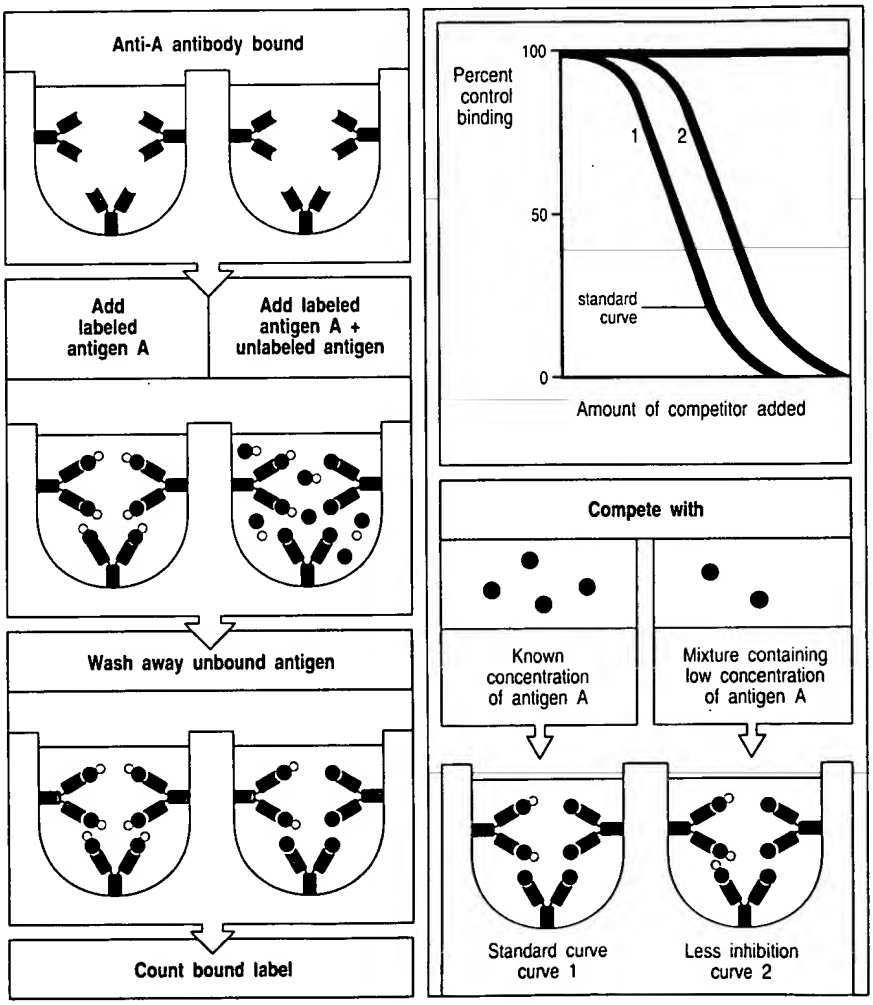



**Fig. 2.7 Competitive inhibition assay for antigen in unknown samples.** A fixed amount of unlabeled antibody is attached to a set of wells, and a standard reference preparation of a labeled antigen is bound to it. Unlabeled standard or test samples are then added in varying amounts and the displacement of labeled antigen is measured, generating characteristic inhibition curves. A standard curve is obtained using known amounts of unlabeled antigen identical to that used as the labeled species, and comparison with this curve allows the amount of antigen in unknown samples to be calculated. The green line on the graph represents a sample lacking any substance that reacts with anti-A antibodies.

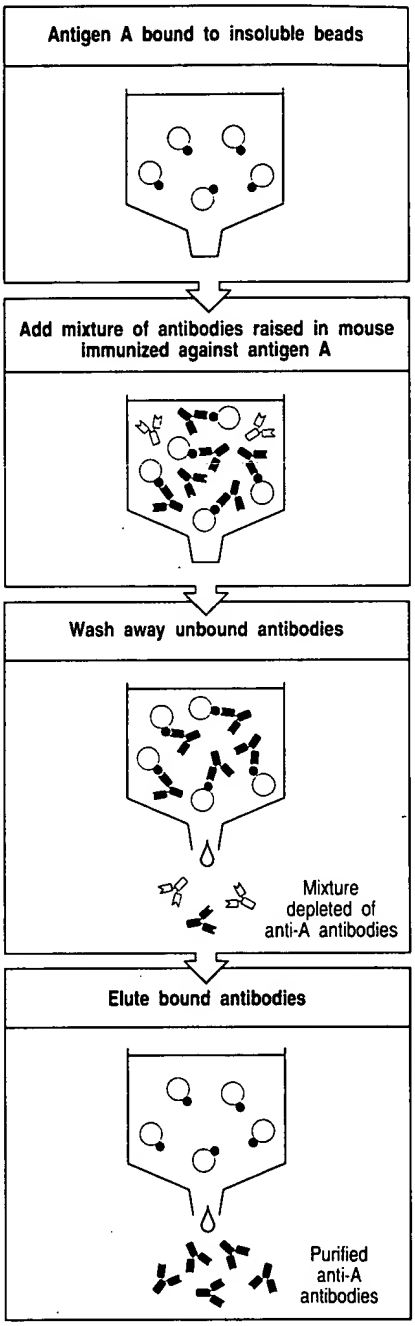



**Fig. 2.8 Affinity chr mat graphy uses antigen:antib dy binding t purify antibodies r antigens.** To purify a specific antibody from serum, antigen is attached to an insoluble matrix, such as chromatography beads, and the serum is passed over the matrix. The specific antibody binds, while other antibodies and proteins are washed away. Specific antibody is then eluted by altering the pH, which can usually disrupt antigen:antibody bonds. In this way, antibodies can be purified from highly complex mixtures of proteins. Antigens can be purified in the same way on beads coupled to antibody (not shown).

of the total protein in an antiserum, even after repeated immunizations. Therefore, antibody must be purified before it can be labeled. Specific antibody can be isolated from an antiserum using **affinity chromatography**, which exploits the specific binding of antibody to antigen held on a solid matrix (Fig. 2.8). Antigen is bound covalently to small, chemically reactive beads, which are loaded into a column, and the antiserum is allowed to pass over the beads. The specific antibodies bind, while all the other proteins in the serum, including antibodies to other substances, can be washed away. The specific antibodies are then eluted, typically by lowering the pH to 2.5 or raising it to greater than 11. This demonstrates that antibodies bind stably under physiological conditions of salt concentration, temperature, and pH, but that the bonds are non-covalent since the binding is reversible. Affinity chromatography can also be used to purify antigens from complex mixtures by coating the beads with specific antibody. The technique is known as affinity chromatography because it separates molecules on the basis of their affinity for one another.

## 2-8 Antibody binding can be detected by changes in the physical state of the antigen.

The direct measurement of antibody binding to antigen is used in most quantitative serological assays. However, some important assays are based on the ability of antibody binding to alter the physical state of the antigen it binds to. These **secondary interactions** can be detected in a variety of ways. For instance, when the antigen is displayed on the surface of a large particle like a bacterium, antibodies can cause the bacteria to clump or **agglutinate**. The same principle applies to the reactions used in blood typing, only here the target antigens are on the surface of red blood cells and the clumping reaction caused by antibodies against them is called **hemagglutination** (from the Greek, *haima*, blood).

This procedure is used to determine the ABO blood group of blood donors and transfusion recipients by inducing clumping or agglutination with antibodies (agglutinins) anti-A or anti-B that bind to the A or B blood group substances respectively (Fig. 2.9). These blood-group antigens are arrayed in many copies on the surface of the red blood cell, causing the cells to agglutinate when crosslinked by antibodies. Since agglutination involves the crosslinking of blood cells by simultaneous binding of antibody molecules to identical antigens on different cells, this reaction demonstrates that each antibody molecule has at least two identical antigen-binding sites.

When sufficient quantities of antibody are mixed with soluble macromolecular antigens, a visible precipitate consisting of large aggregates of antigen crosslinked by antibody molecules can form. The amount of

Related to Part I

**Fig. 2.9 Hemagglutination is used to type blood groups and match compatible donors and recipients for blood transfusion.** Common gut bacteria bear antigens that are similar or identical to blood group antigens, and these stimulate the formation of antibodies to these antigens in individuals who do not bear the corresponding antigen on their own red blood cells (left column); thus, type O individuals, who lack A and B, have both anti-A and anti-B antibodies, while type AB individuals have neither. The pattern of agglutination of the red blood cells of a transfusion donor or recipient with anti-A and anti-B antibodies reveals the individual's ABO blood group. Before transfusion, the serum of the recipient is also tested for antibodies that agglutinate the red blood cells of the donor, and vice versa, a procedure called a cross-match, which may detect potentially harmful antibodies to other blood groups that are not part of the ABO system.

Related to Part II

| Serum from individuals of type | Red blood cells from individuals of type: O | A | B | AB |
|---|---|---|---|---|
| | Express the carbohydrate structures: R-GlcNAc-Gal (Fuc) | R-GlcNAc-Gal-GalNAc (Fuc) | R-GlcNAc-Gal-Gal (Fuc) | R-GlcNAc-Gal-GalNAc (Fuc) / R-GlcNAc-Gal-Gal (Fuc) |
| O — Anti-A and anti-B antibodies | no agglutination | agglutination | agglutination | agglutination |
| A — Anti-B antibodies | no agglutination | no agglutination | agglutination | agglutination |
| B — Anti-A antibodies | no agglutination | agglutination | no agglutination | agglutination |
| AB — No antibodies to A or B | no agglutination | no agglutination | no agglutination | no agglutination |

precipitate depends on the amounts of antigen and antibody, and on the ratio between them (Fig. 2.10). This **precipitin reaction** provided the first quantitative assay for antibody but is now seldom used in immunology. However, it is important to understand the interaction of antigen with antibody that leads to this reaction, as the production of antigen:antibody complexes (**immune complexes**) *in vivo* occurs in almost all immune responses and occasionally can cause significant pathology (see Chapters 11 and 12).

In the precipitin reaction, varying amounts of soluble antigen are added to a fixed amount of serum containing antibody. As the amount of antigen added increases, the amount of precipitate generated also increases up to a maximum and then declines (see Fig. 2.10). When small amounts of antigen are added, antigen:antibody complexes are formed under conditions of antibody excess so that each molecule of antigen is bound extensively by antibody and crosslinked to other molecules of antigen. When large amounts of antigen are added, only small antigen:antibody complexes can form and these are often soluble in this zone of antigen excess. Between these two zones, all of the antigen and antibody is found in the precipitate, generating a zone of equivalence. At equivalence,

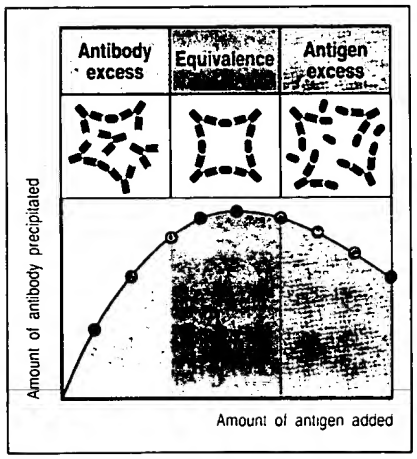



**Fig. 2.10 Antibody can precipitate soluble antigen to generate a precipitin curve.** Different amounts of antigen are added to a fixed amount of antibody, and precipitates form by antibody crosslinking of antigen molecules. The precipitate is recovered and the amount of precipitated antibody measured, while the supernatant is tested for residual antigen or antibody. This defines zones of antibody excess, equivalence, and antigen excess. At equivalence, the largest antigen:antibody complexes form. In the zone of antigen excess, some of the immune complexes are too small to precipitate. These soluble immune complexes can cause pathological damage to small blood vessels when they form *in vivo* (see Chapter 12).

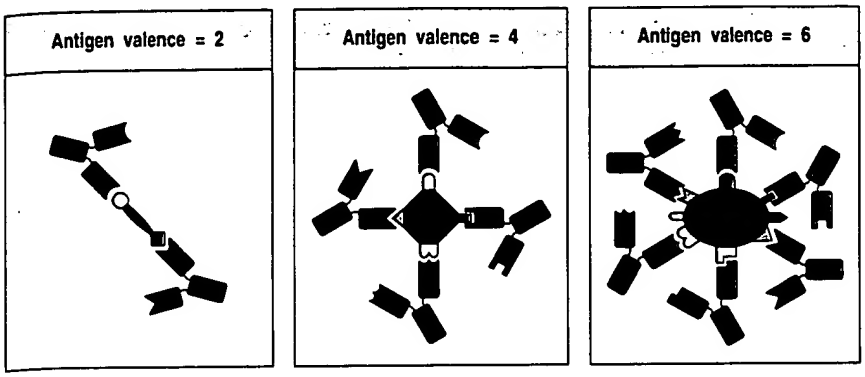



**Fig. 2.11 Different antibodies bind to distinct epitopes on an antigen molecule.** The surface of an antigen possesses many potential antigenic determinants or epitopes, distinct sites to which an antibody can bind. The number of antibody molecules that can bind to a molecule of antigen at one time defines the antigen's valence. Steric considerations can limit the number of different antibodies that bind to the surface of an antigen at any one time (right panel) so that the number of epitopes on an antigen (here 8) is always greater than or equal to its valence (here 6).

very large lattices of antigen and antibody are formed by crosslinking. The small, soluble immune complexes formed in the zone of antigen excess are the cause of pathology *in vivo*.

Antibody can only precipitate antigen molecules that have several antibody-binding sites, so that large antigen:antibody complexes can be formed. Macromolecular antigens have a complex surface to which antibodies of many different specificities can bind. The site to which each distinct antibody molecule binds is called an **antigenic determinant** or an **epitope**. As a result of the complexity of macromolecular surfaces, a single molecule of antigen has many different epitopes. However, steric considerations limit the number of distinct antibody molecules that can bind to a molecule of antigen at any one time, since antibody molecules binding to epitopes that partially overlap will compete for binding. For this reason, the **valence** of an antigen, which is the number of antibody molecules that can bind to a molecule of the antigen at saturation, is almost always less than the number of epitopes on the antigen (Fig. 2.11).

The precipitation of antigen by antibody can be exploited to characterize the antigen:antibody mixtures by carrying out the reaction in a clear gel. When antigen is placed in one well cut in the gel and the antibody is placed in an adjacent well, they diffuse into the gel and form a line of visible precipitate where they meet at equivalence (Fig. 2.12). The same principle is used in other assays that we shall learn about in subsequent sections.

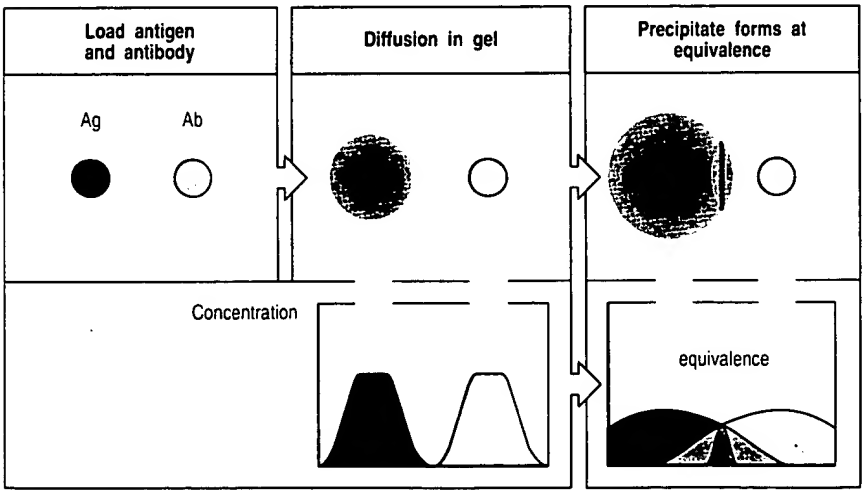


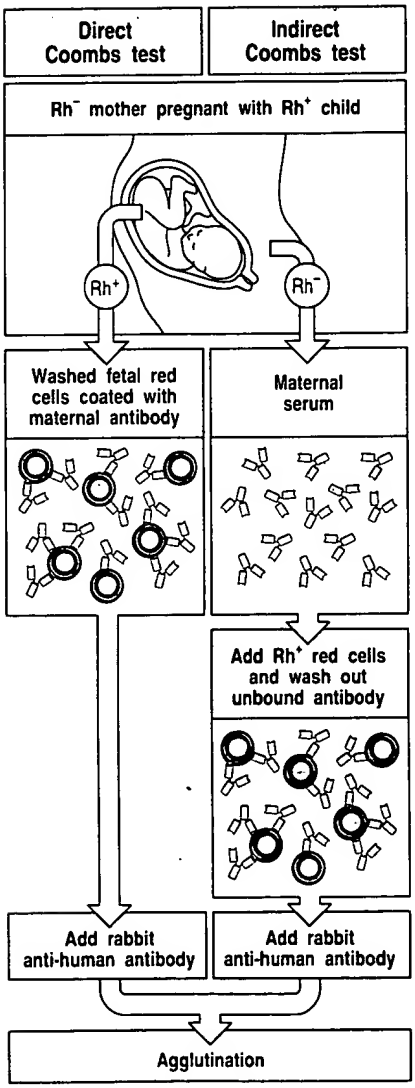

**Fig. 2.12 Ouchterlony gel-diffusion assay for antigen:antibody binding.** Antigen and antibody are placed in different wells of an agar gel. They diffuse toward each other and precipitate where they meet at equivalence, forming a visible band. When serum samples in two wells are tested against the same well of antigen, the relatedness of the samples can be determined by the shape of the precipitin line formed (not shown).

## 2-9 Anti-immunoglobulin antibodies are a useful tool for detecting bound antibody molecules.

As we learned in Section 2-7, antibody can be detected by the direct binding of labeled antibody to antigen coated on plastic surfaces. A more general approach that avoids the need to label each preparation of antibody molecule is to detect bound, unlabeled antibody with a labeled antibody specific for all immunoglobulins. Immunoglobulins, like all other proteins, are immunogenic when used to immunize individuals of another species. The majority of **anti-immunoglobulin antibodies** raised in this way recognize conserved features of antibodies shared by all immunoglobulin molecules. These anti-immunoglobulin antibodies can be purified using affinity chromatography, then labeled, and used as a general probe for bound antibody. Anti-immunoglobulin antibodies were first developed by Robin Coombs to study **hemolytic disease of the newborn**, or **erythroblastosis fetalis**, and the test for this disease is still called the Coombs test. Hemolytic disease of the newborn occurs when a mother makes IgG antibodies specific for the **Rhesus** or **Rh blood group antigen** expressed on the red blood cells of her fetus. Rh-negative mothers make these antibodies when they are exposed to Rh-positive fetal red blood cells bearing the paternally inherited Rh antigen. Maternal IgG antibodies are normally transported across the placenta to the fetus where they protect the newborn infant against infection. However, IgG anti-Rh antibodies coat the fetal red blood cells, which are then destroyed by phagocytic cells in the liver, causing a hemolytic anemia in the fetus and newborn infant.

Related to Part II

Related to Part V




Since the Rh antigens are widely spaced on the red blood cell surface, the IgG anti-Rh antibodies cannot fix complement and cause lysis of red blood cells *in vitro*. Furthermore, for reasons that are not fully understood, antibodies to Rh blood group antigens do not agglutinate red blood cells as do antibodies to the ABO blood group antigens. Thus detecting these antibodies was difficult until anti-human immunoglobulin antibodies were developed. With these, maternal IgG antibodies bound to the fetal red blood cells can be detected after washing the cells to remove unbound immunoglobulin in the serum that interferes with detection of bound antibody. Adding anti-human immunoglobulin antibodies to the washed fetal red blood cells agglutinates any cells to which maternal antibodies are bound. This is the **direct Coombs test** (Fig. 2.13), so called because it directly detects antibody bound to the surface of the patient's red blood cells. An **indirect Coombs test** is used to detect non-agglutinating anti-Rh antibody in serum; the serum is first incubated with Rh-positive red blood cells, which bind the anti-Rh antibody, after which the antibody-coated cells are washed to remove unbound immunoglobulin and are then agglutinated with anti-immunoglobulin antibody (see Fig. 2.13). The indirect Coombs test allows

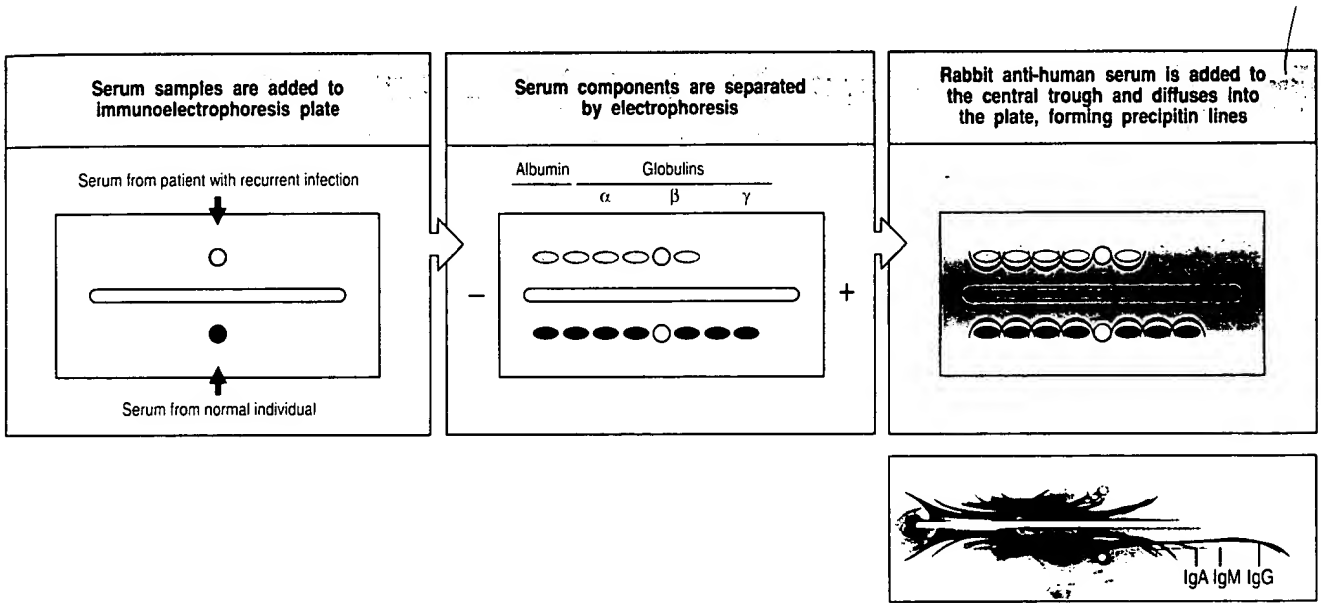
**Fig. 2.13 The Coombs direct and indirect anti-globulin tests for antibody to red blood cell antigens.** A Rh⁻ mother of a Rh⁺ fetus can become immunized to fetal red blood cells that enter the maternal circulation at the time of delivery. In a subsequent pregnancy with a Rh⁺ fetus, IgG anti-Rh antibodies can cross the placenta and damage the fetal red blood cells. In contrast to anti-Rh antibodies, anti-ABO antibodies are of the IgM isotype and cannot cross the placenta, and so do not cause harm. Anti-Rh antibodies do not agglutinate red blood cells but their presence on the fetal red cell surface can be shown by washing away unbound immunoglobulin and then adding antibody to human immuno- globulin, which agglutinates the antibody-coated cells. Anti-Rh antibodies can be detected in the mother's serum in an indirect Coombs test; the serum is incubated with Rh⁺ red blood cells, and once the antibody binds, the red cells are treated as in the direct Coombs test.

Rh incompatibilities that might lead to hemolytic disease of the newborn to be detected and this knowledge allows the disease to be prevented, as we shall see in Chapter 9.

Anti-immunoglobulin antisera have found many uses in clinical medicine and biological research since their introduction. Labeled anti-immunoglobulin antibodies can be used in radioimmunoassay or ELISA to detect binding of unlabeled antibody to antigen-coated plates. The ability of anti-immunoglobulins to react with antibodies of all specificities demonstrates that antibody molecules have constant features recognizable by the anti-immunoglobulin, in addition to the variability required for antibodies to discriminate between a myriad of antigens. The presence of both constant and variable features in one protein posed a genetic puzzle for immunologists, the solution to which is described in Chapter 3.

Some anti-immunoglobulin antibodies made in rabbits react with only a subset of human immunoglobulin molecules. It was this property of anti-immunoglobulin antibodies that led to the discovery that several distinct sets of antibodies, the immunoglobulin isotypes, are present in human serum. The different immunoglobulin isotypes can be seen, along with other serum proteins, by combining electrophoresis, which separates the proteins by charge, with immunodiffusion to detect individual proteins as precipitin arcs. This is achieved by placing an antiserum against whole human serum in a trough that is cut parallel to the direction of electrophoresis, so that each antibody forms an arc of precipitation with a particular serum protein. This technique is called **immunoelectrophoresis** (Fig. 2.14).

Relate to Part




Relate to Part

**Fig. 2.14 Immunoelectrophoresis reveals the presence of several distinct immunoglobulin isotypes in normal human serum.** Serum samples from a normal control and from a patient with recurrent bacterial infection caused by an absence of antibody production, as reflected in an absence of gamma globulins, are separated by electrophoresis on an agar coated slide. Antiserum raised against whole normal human serum and containing antibodies to many of its different proteins is put in a trough down the middle, and each antibody forms an arc of precipitation with the protein it recognizes (see Fig. 2.12). The position of each arc is determined by the electrophoretic mobility of the serum protein; immunoglobulins migrate to the gamma globulin region of the gel. The absence of immunoglobulins in a patient who has X-linked agammaglobulinemia, a form of immune deficiency in which no antibodies of any isotype are formed, is shown in the photograph at the bottom, where several arcs are missing from the patient's serum (upper set). These are IgM, IgA, and several subclasses of IgG, each recognized in normal serum (lower set) by antibodies in the antiserum against human serum proteins. Photograph from the collection of the late C A Janeway Snr.

Serum from rare individuals, who demonstrate increased susceptibility to infection, show no precipitin lines in the region where precipitin arcs for the immunoglobulin isotypes now known as IgM, IgA, and several subclasses of IgG are normally detected. As proteins migrating in this region of a serum protein electrophoresis were originally called **gamma globulins**, the failure to make immunoglobulins, which is the result of a single-gene defect on the X chromosome and so occurs mainly in males, is called **X-linked agammaglobulinemia**; its cause has recently been discovered, as we shall see in Chapter 10.

Anti-immunoglobulins specific for each isotype can be produced by immunizing an animal of a different species with a pure preparation of one isotype and then removing those antibodies that cross-react with immunoglobulins of other isotypes using affinity chromatography (see Fig. 2.8). Anti-isotype antibodies can be used to measure how much antibody of a particular isotype in an anti-serum reacts with a given antigen. This reaction is particularly important for detecting small amounts of specific IgE antibodies, which are responsible for most allergies. IgE binding to an antigen correlates with allergic reactions to that antigen.

Related to Part II

An alternative approach to detecting bound antibodies exploits bacterial proteins that bind to immunoglobulins with high affinity and specificity. One of these, **Protein A** from the bacterium *Staphylococcus aureus*, has been exploited widely in immunology for the affinity purification of immunoglobulin and for detection of bound antibody.

The use of standard second reagents such as labeled anti-immunoglobulin or Protein A to detect antibody bound specifically to its antigen allows great savings in reagent labeling costs, and also provides a standard detection system so that results in different assays can be compared directly.

## 2-10 Antisera contain heterogeneous populations of antibody molecules.

The antibodies generated in a natural immune response or after immunization in the laboratory are a mixture of molecules of different specificities and affinities. Some of this heterogeneity results from the production of antibodies that bind to different epitopes on the immunizing antigen, but even antibodies directed at a single antigenic determinant such as a hapten can be markedly heterogeneous. This heterogeneity is detectable by **isoelectric focusing**. In this technique, proteins are separated on the basis of their isoelectric point, the pH at which their net charge is zero. By electrophoresing proteins in a pH gradient for long enough, each molecule migrates along the pH gradient until it reaches the pH at which it is neutral, and is thus concentrated (focused) at that point. When antiserum containing anti-hapten antibodies is treated in this way and then transferred to a solid support such as nitrocellulose paper, the anti-hapten antibodies can be detected by their ability to bind labeled hapten (Fig. 2.15). The binding of antibodies of varying isoelectric points to the hapten shows that even antibodies that bind the same antigenic determinant are heterogeneous.

Antisera are valuable for many biological purposes but they have certain disadvantages that relate to the heterogeneity of the antibodies they contain. First, each antiserum is different from all other antisera, even if raised in a genetically identical animal using the identical preparation of antigen and the same immunization protocol. Second, antisera can only be produced in limited volumes, and thus it is impossible to use the identical serological reagent in a long or complex series of experiments or clinical tests. Finally, even antibodies purified by affinity chromatography (see Section 2-7) may include minor populations of antibodies that give

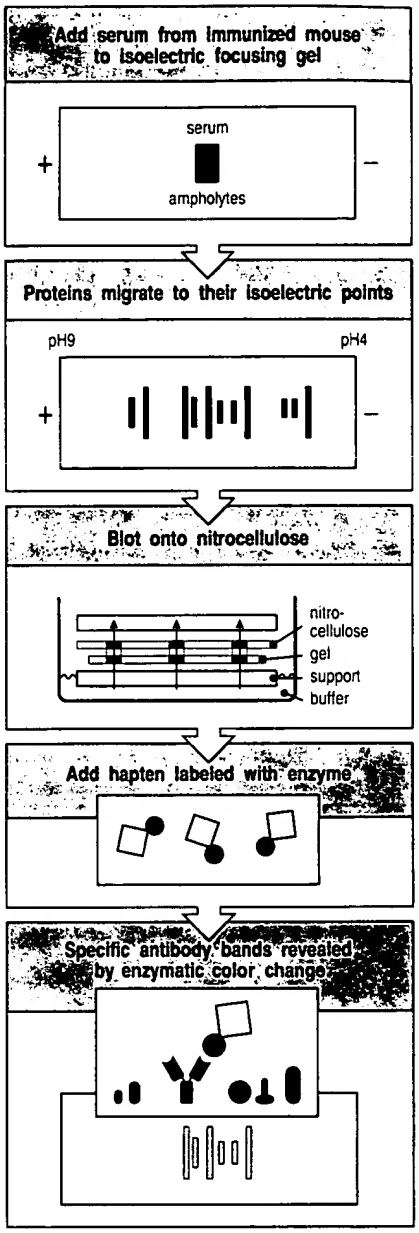



**Fig. 2.15 Isoelectric f cusing f antiserum reveals the heter geneity of antibodies specific f r a given antigen.** The heterogeneity of antibodies specific for a hapten can be shown by gel electrophoresis of antiserum in a pH gradient generated by ampholytes. The serum proteins migrate to a pH equivalent to their isoelectric point, where they become uncharged and cease to migrate. The proteins are transferred to nitrocellulose paper, which is then treated with the enzyme-coupled hapten, which binds the hapten-specific antibodies in the antiserum. These antibodies are then detected by an enzymatic reaction, which produces a colored product from a colorless substrate, as in ELISA. Even antibodies to a single hapten can be very heterogeneous, as seen here, because of differences in their amino acid sequence.

unexpecied cross-reactions, which confound the analysis of experiments. For this reason, an unlimited supply of antibody molecules of homogeneous structure and known specificity would be very desirable. This has been achieved through the production of monoclonal antibodies from hybrid antibody-forming cells or, more recently, by genetic engineering, as we shall see in the next section.

Relate to Part

## 2-11 Monoclonal antibodies have a homogeneous structure and can be produced by cell fusion or by genetic engineering.

Biochemists in search of a homogeneous preparation of antibody that they could subject to detailed chemical analysis turned first to proteins produced by patients with multiple myeloma, a common tumor of plasma cells. It was known that antibodies are normally produced by plasma cells and since this disease is associated with the presence of large amounts of a homogeneous gamma globulin called a **myeloma protein** in the patient's serum, it seemed likely that myeloma proteins would serve as models for normal antibody molecules. Thus, much of the early knowledge of antibody structure came from studies on myeloma proteins. These studies showed that monoclonal antibodies could be obtained from immortalized plasma cells. However the antigen-specificity of most myeloma proteins was unknown, which limited their usefulness as objects of study, or as immunological tools.

This problem was solved by Georges Köhler and Cesar Milstein, who devised a technique for producing a homogeneous population of antibodies of known antigenic specificity. They did this by fusing spleen cells from an immunized mouse to cells of a mouse myeloma to produce hybrid cells that both proliferated indefinitely and secreted antibody specific for the antigen used to immunize the spleen cell donor. The spleen cell provides the ability to make specific antibody, while the myeloma cell provides the ability to grow indefinitely in culture and secrete immunoglobulin continuously. By using a myeloma cell partner that produces no antibody proteins itself, the antibody produced by the hybrid cells comes only from the immune spleen cell partner. After fusion, the hybrid cells are selected using drugs that kill the myeloma parental cell, while the unfused parental spleen cells have a limited lifespan and soon die, so that only hybrid myeloma cell lines or **hybridomas** survive. Those hybridomas producing antibody of the desired specificity are then identified and cloned by regrowing the cultures from single cells. Since each hybridoma is a **clone** derived from fusion with a single B cell, all the antibody

molecules it produces are identical in structure, including their antigen-binding site and isotype. Such antibodies are therefore called **monoclonal antibodies** (Fig. 2.16). This technology has revolutionized the use of antibodies by providing a limitless supply of antibody of a single and known specificity and a homogeneous structure. Monoclonal antibodies are now used in most serological assays as diagnostic probes, and as therapeutic agents.

Recently, a novel technique for producing antibody-like molecules has been introduced. Gene segments encoding the antigen-binding variable or V domains of antibodies are fused to genes encoding the coat protein of a bacteriophage. Bacteriophage containing such gene fusions are used to infect bacteria, and the resulting phage particles have coats that express the antibody-like fusion protein, with the antigen-binding domain displayed on the outside of the bacteriophage. A collection of recombinant phage, each displaying a different antigen-binding domain on its surface, is known as a **phage display library**. In much the same way that antibodies specific for a particular antigen can be isolated from a complex mixture using affinity chromatography (see Section 2-7), phage expressing antigen-binding domains specific for a particular antigen can be isolated by selecting the phage in the library for binding to that antigen. The phage particles that bind are recovered and used to infect fresh bacteria. Each phage isolated in this way will produce a monoclonal antigen-binding particle analogous to a monoclonal antibody (Fig. 2.17). The genes encoding the antigen-binding site, which are unique to each phage, can then be recovered from the phage DNA and used to construct genes for a complete antibody molecule by joining them to gene segments that encode the invariant parts of an antibody. When these reconstructed antibody genes are introduced into a suitable host cell line, such as the non-antibody producing myeloma cells used for hybridomas, the transfected cells secrete antibodies with all the desirable characteristics of monoclonal antibodies produced from hybridomas. This technique may ultimately replace the traditional route of cell fusion for production of monoclonal antibodies.

Related to Part II

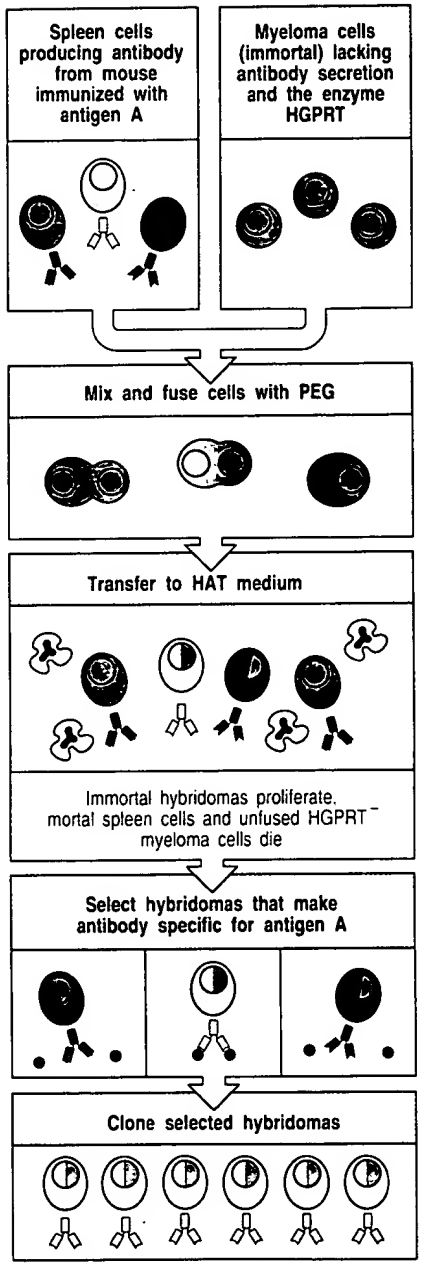



**Fig. 2.16 The production of monoclonal antibodies.** Mice are immunized with antigen A and given an intravenous booster immunization three days before they are killed in order to produce a large population of spleen cells secreting specific antibody. Spleen cells die after a few days in culture. In order to produce a continuous source of antibody they are fused with immortal myeloma cells using polyethylene glycol (PEG) to produce a hybrid cell line called a hybridoma. The myeloma cells are selected beforehand to ensure that they are not secreting antibody themselves and that they are sensitive to the hypoxanthine-aminopterin-thymidine (HAT) medium that is used to select hybrid cells because they lack the enzyme hypoxanthine:guanine phosphoribosyl transferase (HGPRT). The HGPRT gene contributed by the spleen cell allows hybrid cells to survive in the HAT medium, and only hybrid cells can grow continuously in culture because of the malignant potential contributed by the myeloma cells. Therefore, unfused myeloma cells and unfused spleen cells die in the HAT medium, as shown here by cells with dark, irregular nuclei. Individual hybridomas are then screened for antibody production, and cells that make antibody of the desired specificity are cloned by growing them up from a single antibody-producing cell. The cloned hybridoma cells are grown in bulk culture to produce large amounts of antibody. As each hybridoma is descended from a single cell, all the cells of a hybridoma cell line make the same antibody molecule, which is called a monoclonal antibody.

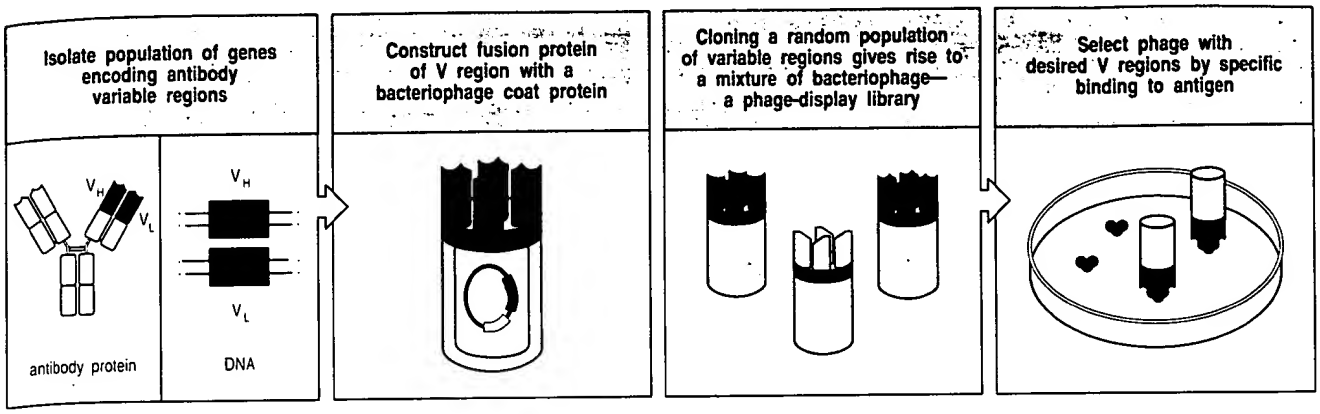



**Fig. 2.17 The production of antibodies by genetic engineering.** Short primers to consensus sequences in heavy- and light-chain variable or V regions of immunoglobulin genes are used to generate a library of heavy- and light-chain V-region cDNAs by the polymerase chain reaction (see Fig. 2.44) using spleen mRNA as the starting material. These heavy- and light-chain V-region genes are cloned randomly into a filamentous phage such that each phage expresses one heavy- and one light-chain V region as a surface fusion protein with antibody-like properties. The resulting phage display library is expanded in bacteria, and the phage are then bound to a surface coated with antigen. The unbound phage are washed away, while the bound phage are recovered and again bound to antigen. After a few cycles only specific high-affinity antigen-binding phage are left. These can be used like antibody molecules, or their V genes can be recovered and engineered into antibody genes to produce genetically engineered antibody molecules (not shown). This technology may replace the hybridoma technology for producing monoclonal antibodies and has the advantage that any species can be used as the source of the initial mRNA.

Relate to Part I

## 2-12 The affinity of an antibody can be determined directly by measuring binding to small monovalent ligands.

The **affinity** of an antibody is the strength of binding of a monovalent ligand to a single antigen-binding site. The affinity of an antibody that binds small antigens, such as haptens that can diffuse freely across a dialysis membrane, can be determined directly by the technique of **equilibrium dialysis**. A known amount of antibody, whose molecules are too large to cross a dialysis membrane, is placed in a dialysis bag and offered varying amounts of antigen. Molecules of antigen that bind to the antibody are no longer free to diffuse across the dialysis membrane, so only the unbound molecules of antigen equilibrate across it. By measuring the concentration of antigen inside the bag and in the surrounding fluid, one can determine the amount of the antigen that is bound as well as the amount that is free when equilibrium has been achieved. Given that the amount of antibody present is known, the affinity of the antibody and the number of specific binding sites for the antigen per molecule of antibody can be determined from this information. The data is usually analyzed using **Scatchard analysis** (Fig. 2.18); such analyses were used to demonstrate that a molecule of IgG has two identical antigen-binding sites.

While affinity measures the strength of binding of an antigenic determinant to a single antigen-binding site, an antibody reacting with an antigen that has multiple identical epitopes or with the surface of a pathogen will often bind the same molecule or particle with both of its antigen-binding sites. This increases the apparent strength of binding, since both binding sites must release at the same time in order for the two molecules to dissociate. This is often referred to as **cooperativity** in binding, but it should not be confused with the cooperative binding found in a protein such as hemoglobin in which binding of ligand at one site enhances the affinity of a second binding site for its ligand. The overall strength of binding of an antibody molecule to an antigen or particle is called its

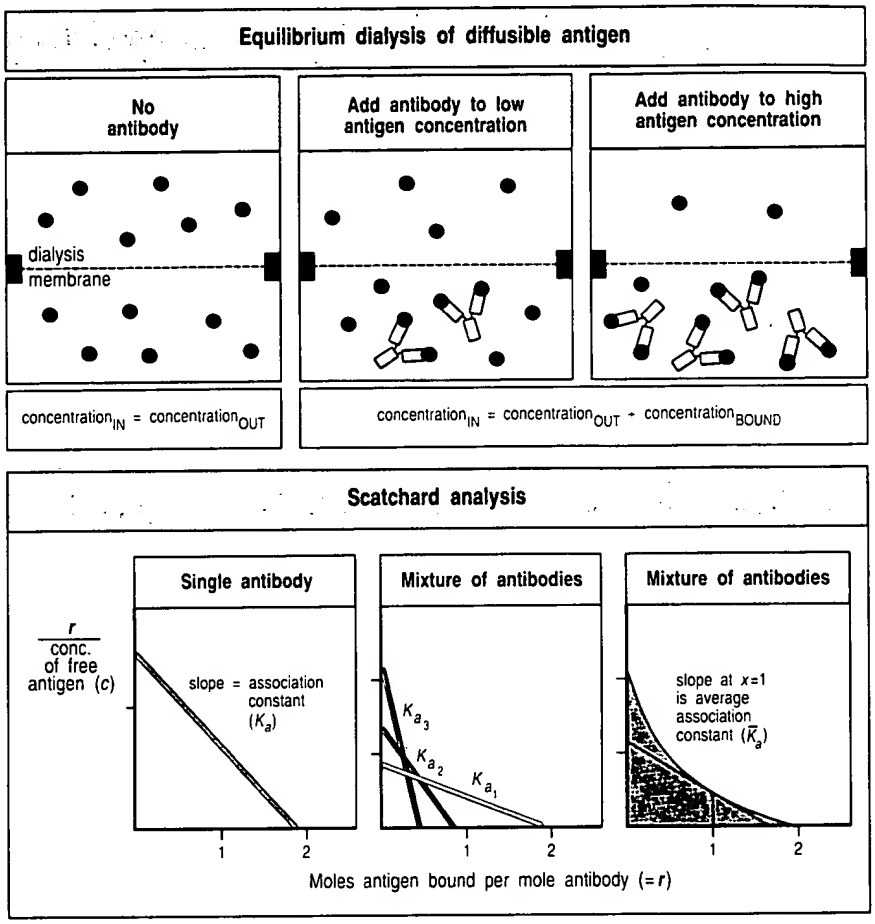



**Fig. 2.18 The affinity and valence of an antibody can be determined by equilibrium dialysis.** A known amount of antibody is placed in the bottom half of a dialysis chamber and exposed to different amounts of a diffusible monovalent antigen, such as a hapten. At each concentration of antigen added, the fraction of the antigen bound is determined from the difference in concentration of total antigen in the top and bottom chambers. This information can be transformed into a Scatchard plot as shown here. In Scatchard analysis, the ratio of *r*/c, where *r* = moles of antigen bound per mole of antibody and c = molar concentration of free antigen, is plotted against *r*. The number of binding sites per antibody molecule can be determined from the value of *r* at infinite free-antigen concentration, where *r*/free = 0, in other words at the x-axis intercept. The analysis of a monoclonal IgG antibody molecule in which there are two identical antigen-binding sites per molecule is shown in the left panel. The slope of the line is determined by the affinity of the antibody molecule for its antigen; if all the antibody molecules in a preparation are identical, as for this monoclonal antibody, then a straight line is obtained whose slope is equal to $-K_a$, where $K_a$ is the association (or affinity) constant and the dissociation constant $K_d = 1/K_a$. However, antisera raised even against a simple antigenic determinant such as a hapten containing heterogeneous populations of antibody molecules (see Section 2-10). Each antibody molecule would, if isolated, make up part of the total and give a straight line whose x-axis intercept is less than two, as it contains only a fraction of the total binding sites in the population (middle panel). As a mixture, they give curved lines with an x-axis intercept of two for which an average affinity ($\overline{K}_a$) can be determined from the slope of this line at a concentration of antigen where 50% of the sites are bound, or at x = 1 (right panel). The association constant determines the equilibrium state of the reaction Ag + Ab = Ag:Ab, where antigen = Ag and antibody = Ab, and $K_a$ = [Ag:Ab]/[Ag][Ab]. This constant reflects the 'on' and 'off' rates for antigen binding to the antibody; with small antigens like haptens, binding is usually as rapid as diffusion allows, while differences in off rates determine the affinity constant. However, with larger antigens the 'on' rate may also vary as the interaction becomes more complex.

Related to Part II

**avidity** (Fig. 2.19). For IgG antibodies, bivalent binding can significantly increase avidity; in IgM antibodies, which have ten identical antigen-binding sites, the affinity of each site for a monovalent antigen is usually quite low, but the avidity of binding of the whole antibody to a surface such as a bacterium that displays multiple identical epitopes can be very high.

## 2-13 Antibodies can be used to identify antigen in cells, tissues, and complex mixtures of substances.

Since antibodies bind stably and specifically to antigen, they are invaluable as probes for identifying a particular molecule in cells, tissues, or biological fluids. They are used in this way to study a wide range of biological processes and clinical conditions. In this section, a few techniques that are used to study the immune system, as well as in cell biology generally, will be described; a complete treatment of this subject can be found in any of the excellent methodology books available.

Antibody molecules can be used to locate their target molecules accurately in single cells or in tissue sections by a variety of different labeling techniques. As in all serological tests, the antibody binds stably to its antigen, allowing unbound antibody to be removed by thorough washing. As antibodies to proteins recognize the surface features of the native, folded protein, the native structure of the protein being sought usually

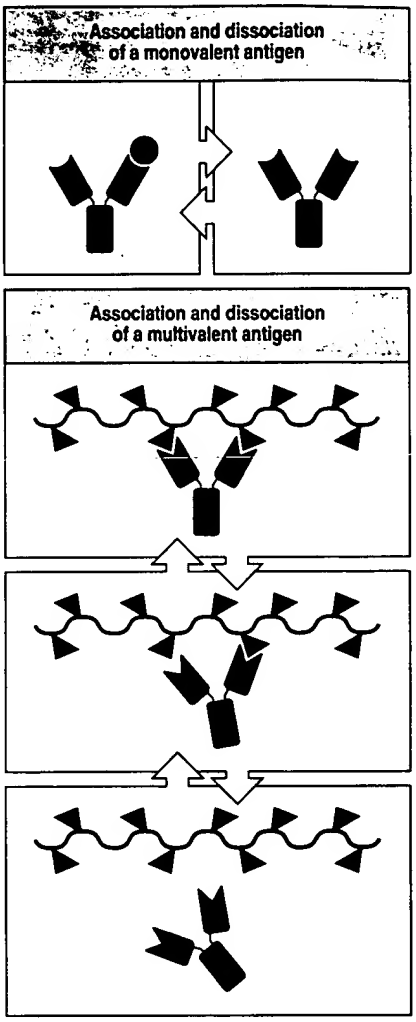



**Fig. 2.19 The avidity of an antibody is its strength of binding to intact antigen.** When an IgG antibody binds a ligand with multiple identical epitopes, both binding sites can bind the same molecule or particle. The overall strength of binding, called avidity, is greater than the affinity, the strength of binding of a single site, since both binding sites must dissociate at the same time for the antibody to release the antigen. This property is very important in the binding of antibody to bacteria, which usually have multiple identical epitopes on their surfaces.

needs to be preserved, either by gentle fixation techniques or by using frozen tissue sections that are fixed only after the antibody reaction has been performed. Some antibodies, however, bind proteins even if they are denatured, and such antibodies will bind specifically even to protein in fixed tissue sections.

The bound antibody can be visualized using a variety of sensitive techniques, and the specificity of antibody binding coupled to sensitive detection provides remarkable detail about the structure of cells. One very powerful technique for identifying antibody-bound molecules in cells or tissue sections is **immunofluorescence**, in which a fluorescent dye is attached directly to the specific antibody. More commonly, bound antibody is detected by fluorescent anti-immunoglobulin, a technique known as **indirect immunofluorescence**. The dyes chosen for immunofluorescence are excited by light of one wavelength, usually blue or green, and emit light of a different wavelength in the visible spectrum. By using selective filters, only the light coming from the dye or fluorochrome used is detected in the fluorescence microscope (Fig. 2.20). Although Albert Coons first devised this technique to identify the plasma cell as the source of antibody, it can be used to detect the distribution of any protein. By attaching different dyes to different antibodies, the distribution of two or more molecules can be determined in the same cell or tissue section (see Fig. 2.20). An alternative method of detecting a protein in tissue sections is to use **immunohistochemistry**, in which the antibody is chemically coupled to an enzyme that converts a colorless substrate into a colored reaction product whose deposition can be directly observed under a light microscope. This technique is analogous to the ELISA assay described in Section 2-7.

The recent development of the confocal fluorescent microscope, which uses computer-aided techniques to produce an ultrathin optical section of a cell or tissue, gives very high resolution immunofluorescence microscopy, without the need for elaborate sample preparation. To examine cells at even higher resolution, similar procedures can be applied to ultrathin sections examined in the transmission electron microscope. Antibodies labeled with gold particles of distinct diameter enable two or more proteins to be studied simultaneously. The difficulty with this technique is in staining the ultrathin section, as few molecules of antigen will be present in each section.

In order to raise antibodies against membrane proteins and other cellular structures that are difficult to purify, mice are often immunized with whole cells or crude cell extracts. Antibodies to the individual molecules are then obtained by preparing monoclonal antibodies that bind to the cell used for immunization. To characterize the molecules identified by these antibodies, cells of the same type are labeled with radioisotopes and dissolved in non-ionic detergents that disrupt cell membranes but do not interfere with antigen:antibody interactions. This allows the labeled protein to be isolated by binding to the antibody. The antibody is usually attached to a solid support, such as the beads used in affinity chromatography. Cells can be labeled in two main ways for this **immunoprecipitation analysis**. All of the proteins in a cell

Relate to Part

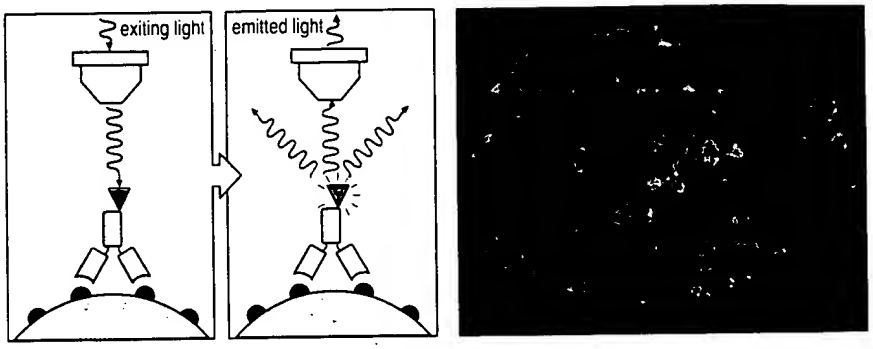



**Fig. 2.20 Immun fluorescence microscopy.** Antibodies labeled with a fluorescent dye such as fluorescein (green triangle) are used to reveal the presence of their corresponding antigens in cells or tissues. The stained cells are examined in a microscope that exposes them to blue or green light to excite the fluorescent dye. The excited dye emits light at a characteristic wavelength, which is captured by viewing the sample through a selective filter. This technique is applied widely in biology to determine the location of molecules in cells and tissues. Different antigens can be detected in tissue sections by labeling antibodies with dyes of distinctive color. Here, antibodies to the protein glutamic acid decarboxylase (GAD) coupled to a green dye are shown to stain the β cells of pancreatic islets of Langerhans, while the α cells, labeled with antibodies to the hormone glucagon coupled with an orange fluorescent dye, do not have this enzyme. GAD is an important autoantigen in diabetes, an autoimmune disease in which the insulin-secreting β cells of the islets of Langerhans are destroyed. Photograph courtesy of M Solimena and P De Camilli.

Related to Part II

can be labeled metabolically by growing the cell in radioactive amino acids that are incorporated into cellular protein (Fig. 2.21). Alternatively, one can label only the cell-surface proteins by radioiodination under conditions that prevent iodine from crossing the plasma membrane and labeling proteins inside the cell, or by a reaction that labels only membrane proteins with biotin, a small molecule that binds covalently with proteins and is detected readily by its reaction with labeled avidin.

Once the labeled proteins have been isolated by the antibody, they can be characterized in several ways. The most common is polyacrylamide gel electrophoresis (PAGE) of the proteins once they have been dissociated from antibody in the strong ionic detergent, sodium dodecyl sulfate (SDS), a technique generally abbreviated as **SDS-PAGE**. SDS binds relatively homogeneously to proteins, conferring a charge that allows the electrophoretic field to drive protein migration through the gel. The rate of

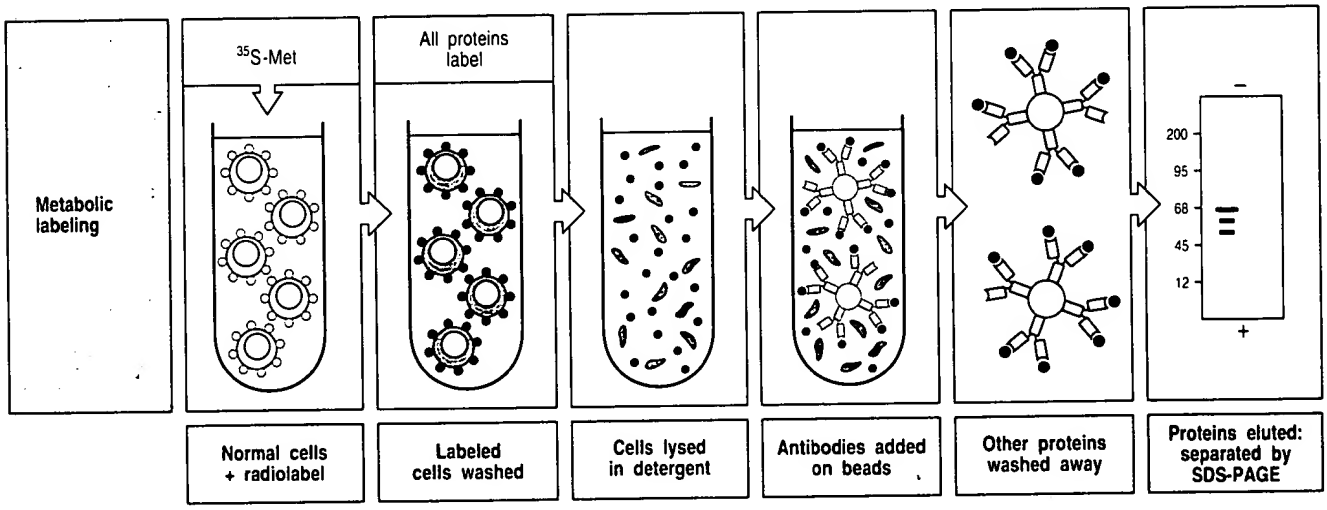



**Fig. 2.21 Cellular proteins reacting with an antibody can be characterized by immunoprecipitation of labeled cell lysates.** All actively synthesized cellular proteins can be labeled metabolically by incubating cells with radioactive amino acids (shown here for methionine) or one can label just the cell-surface proteins by using radioactive iodine in a form that cannot cross the cell membrane or by a reaction with the small molecule biotin, detected by its reaction with labeled avidin (not shown). Cells are lysed with detergent and individual labeled cell-associated proteins can be precipitated with a monoclonal antibody attached to beads. After washing away unbound proteins, the bound protein is eluted in the detergent sodium dodecyl sulfate (SDS), which dissociates it from the antibody and also coats the protein with a strong negative charge, allowing it to migrate according to its size in polyacrylamide gel electrophoresis (PAGE). The positions of the labeled proteins are determined by autoradiography using X-ray film. This technique of SDS-PAGE can be used to determine the molecular weight and subunit composition of a protein. Patterns of protein bands observed using metabolic labeling are usually more complex than those revealed by radioiodination, owing to the presence of precursor forms of the protein (right panel). The mature form of a surface protein can be identified as being the same size as that detected by surface iodination or biotinylation (not shown).

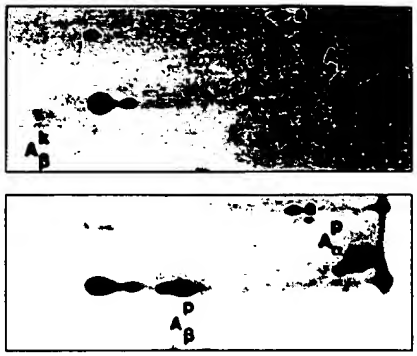


**Fig. 2.22 Tw -dimensional gel electrophoresis f MHC class II molecules.** Proteins in mouse spleen cells have been labeled metabolically (see Fig. 2.20), precipitated with a monoclonal antibody against the mouse MHC class II molecule H2-A, and separated by isoelectric focusing in one direction and SDS-PAGE in a second direction at right angles to the first, hence the term two-dimensional gel. This allows one to distinguish molecules of the same molecular weight on the basis of their charge. The separated proteins are detected using auto-radiography. The MHC molecules are composed of two chains, α and β, and in the different MHC class II molecules these have different isoelectric points (compare upper and lower panels). The MHC genotype of mice is indicated by lower case superscripts (k,p). Actin, a common contaminant, is marked a. Photograph courtesy of J F Babick.

migration is controlled mainly by protein size (see Fig. 2.21). However, this technique does not separate proteins that are of similar size but differ in charge as a result of having different amino acid sequences, such as major histocompatibility complex (MHC) proteins. Proteins of different charge can be separated using isoelectric focusing (see Section 2-10). This technique can be combined with SDS-PAGE in a procedure known as **two-dimensional gel electrophoresis**. For this, the immunoprecipitated protein is eluted in urea, a non-ionic solubilizing agent, and run on an isoelectric focusing gel in one direction in a narrow tube of polyacrylamide. This first-dimensional isoelectric focusing gel is then placed across the top of an SDS-PAGE slab gel, which is then run vertically to separate the proteins by molecular weight. Two-dimensional gel electrophoresis is a powerful technique that allows many hundreds of proteins in a complex mixture to be distinguished from one another (Fig. 2.22).

An alternative approach that avoids the problem of radiolabeling cells is to solubilize all cellular proteins by placing unlabeled cells directly in detergent and running the lysate on SDS-PAGE. The size-separated proteins are then transferred from the gel to a stable support such as nitrocellulose paper. Specific proteins are detected by antibodies (mainly those that react with denatured sequences) and their position revealed by anti-immunoglobulin that is labeled with radioisotopes or an enzyme. This procedure is called **immunoblotting** or **Western blotting**. (The latter term arose because the comparable technique for detecting specific DNA sequences is known as Southern blotting, after Ed Southern who devised it, which in turn provoked the name Northern for blots of size-separated RNA). Western blots are used in many applications in basic research and clinical diagnosis, for example to detect antibodies to different constituents of HIV (Fig. 2.23).

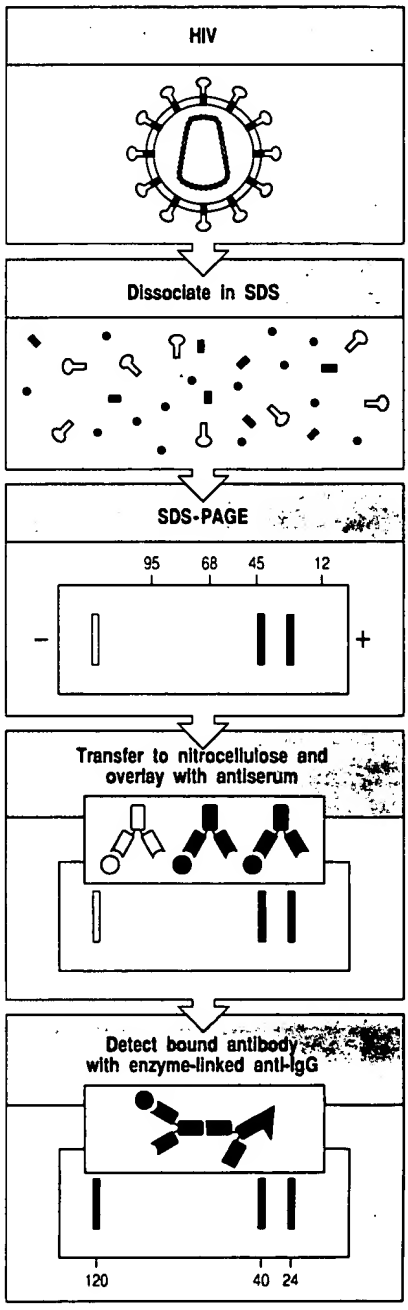



**Fig. 2.23 Western blotting is used to identify antibodies to the human immunodeficiency virus (HIV) in serum from infected individuals.** The virus is dissociated into its constituent proteins by treatment with the detergent SDS, and its proteins separated using SDS-PAGE. The separated proteins are transferred to a nitrocellulose sheet and reacted with the test serum. Anti-HIV antibodies in the serum bind to the various HIV proteins and are detected using enzyme-linked anti-human immunoglobulin, which deposits colored material from a colorless substrate. This general methodology will detect any combination of antibody and antigen and is used widely, although the denaturing effect of SDS means that the technique works most reliably with antibodies that recognize the antigen when it is denatured.

## 2-14 Antibodies can be used to isolate protein antigens for further characterization.

Immunoprecipitation and Western blotting are useful for determining the molecular weight and isoelectric point of a protein. These characteristics can then help to distinguish it from other proteins and provide a means of recognizing it on an electrophoretic gel. The protein's abundance, distribution, and whether, for example, it undergoes changes in molecular weight and isoelectric point as a result of processing within the cell can thus be determined. However, these techniques do not provide a definitive characterization of the protein.

To do this, the protein must be isolated and purified. Antibodies specific for the protein can be used to isolate it using affinity chromatography (see Section 2-7), but this does not usually yield sufficient protein for a full characterization. Often, this small amount of purified protein is used to obtain amino acid sequence information from the protein's amino-terminal end or from proteolytic peptide fragments of the protein. These amino acid sequences are used to generate a set of synthetic oligonucleotides capable of encoding these peptides, which are then used as probes to isolate the gene encoding the protein from either a library of DNA sequences complementary to mRNA (a cDNA library) or a genomic library. The full amino acid sequence of the protein can be deduced from the nucleotide sequence of its cDNA, and this often gives clues to the nature of the protein and its biological properties. The nucleotide sequence of the gene and its regulatory regions can be determined from genomic DNA clones. The gene can be manipulated and introduced into cells by transfection for larger-scale production and functional studies. This approach has been used to characterize many immunologically important proteins, such as the MHC glycoproteins.

Related to Part II

## 2-15 Antibodies can be used to identify genes and their products.

An alternative approach uses antibodies directly to identify and isolate a gene encoding a cell-surface protein. A specific antibody is used to detect the expression of the protein on the surface of a cell type that does not normally express it, after the cell has been transfected with the gene in the form of a cDNA. A suitable cDNA library is prepared from total mRNA isolated from a cell type known to express the protein. The cDNA library is then cloned into special vectors, called expression vectors, which are constructed to allow the genes they carry to be expressed upon transfection into cultured mammalian cells. These vectors drive expression of the gene in the transfected cells without integrating into the host cell DNA. Cells expressing the protein are isolated by binding to antibody (see Section 2-17), and the vector is recovered by lysing the cells (Fig. 2.24).

The vector is then introduced into bacterial cells where it replicates rapidly, and these amplified vectors are used in a second round of transfection in mammalian cells. After several cycles of transfection, isolation, and amplification in bacteria, single colonies of bacteria expressing the vector are picked and used in a final transfection to identify a cloned vector carrying the cDNA of interest, which is then isolated and characterized. This methodology has been used to isolate many genes encoding cell-surface molecules. It cannot, however, be used to isolate genes for proteins that remain within the cell, as these cannot be detected by surface antibody binding, nor can it be used to isolate the genes for proteins that are only expressed on the cell surface as parts of multichain arrays, as these would require the simultaneous expression of several different cDNAs in the same cell to produce a detectable cell-surface molecule.

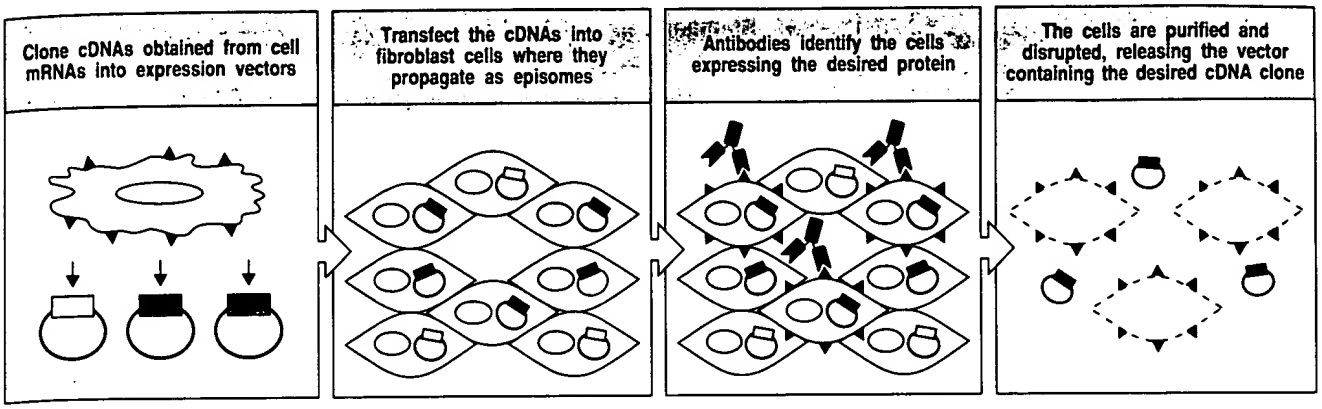



**Fig. 2.24 The gene encoding a cell-surface molecule can be isolated by expressing it in fibroblasts and detecting its protein products with monoclonal antibodies.** Total mRNA from a cell line or tissue expressing the protein is isolated, converted into cDNA, and cloned as a cDNA in a vector designed to direct expression of the cDNA in fibroblasts. The entire cDNA library is used to transfect cultured fibroblasts. Fibroblasts that have taken up cDNA encoding a cell-surface protein express the protein on their surface; they can be isolated by binding a monoclonal antibody against that protein. The vector containing the gene is isolated from these cells and used for more rounds of transfection and re-isolation until uniform positive expression is obtained, ensuring that the correct gene has been isolated. The cDNA insert can then be sequenced to determine the sequence of the protein it encodes and can also be used as the source of material for large-scale expression of the protein for analysis of its structure and function. The method illustrated is limited to cloning genes for single-chain proteins (i.e. those encoded by only one gene) that can be expressed in fibroblasts. It has been used to clone many genes of immunological interest such as that for CD4.

Relate to Part

The converse approach is taken to identify the unknown protein product of a cloned gene. The gene sequence is used to construct synthetic peptides of 10–20 amino acids that are identical to part of the deduced protein sequence, and antibodies are then raised against these peptides by coupling them to carrier proteins; the peptides behave as haptens. These anti-peptide antibodies often bind the native protein and so can be used to identify its distribution in cells and tissues to isolate it and to try to ascertain its function (Fig. 2.25). This approach is often called reverse genetics as it works from gene to phenotype rather than from phenotype to gene, which is the classical genetic approach. The great advantage of reverse genetics over the classical approach is that it does not require a phenotypic genetic trait for identifying a gene. The main disadvantage of reverse genetics is that often there is no mutant phenotype of the gene so that its functional importance may be difficult to infer. However, mutant genes can be produced by the technique of *in vitro* mutagenesis and then inserted into cells and animals in order to test for their effects. In addition, as it is now possible to inactivate genes deliberately in cells and animals, mutant phenotypes can be generated directly from the gene (see Section 2-37).

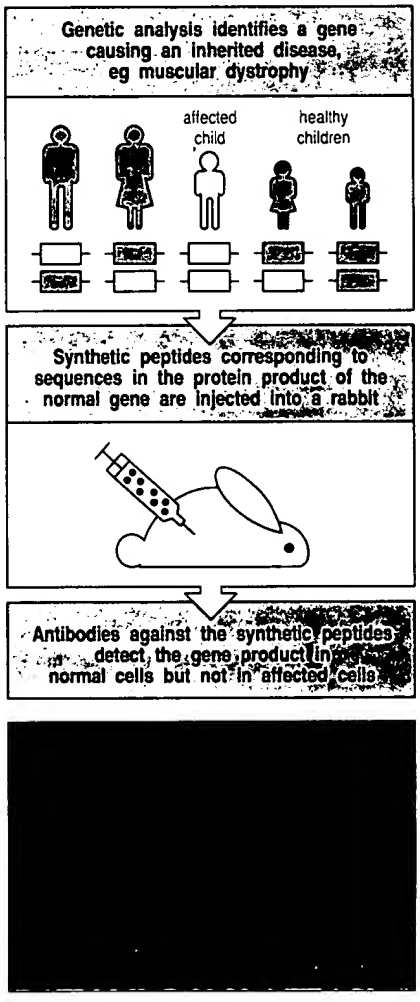



**Fig. 2.25 The use of antibodies to detect the unknown protein product f a known gene is called reverse genetics.** When the gene responsible for a genetic disorder such as Duchenne muscular dystrophy is isolated, the amino acid sequence of the unknown protein product of the gene can be deduced from the nucleotide sequence of the gene and synthetic peptides representing parts of this sequence can be made. Antibodies are raised against these peptides and purified from the antiserum by affinity chromatography on a peptide column (see Fig. 2.8). Labeled antibody is used to stain tissue from individuals with the disease and from unaffected individuals to determine differences in the presence, amount, and distribution of the normal gene product. The product of the dystrophin gene is present in normal mouse skeletal muscle cells, as shown in the bottom panel (red fluorescence); but is missing in the cells of mice bearing the mutation *mdx*, the mouse equivalent of Duchenne muscular dystrophy (not shown). Photograph (x15) courtesy of H G W Lidov and L Kunkel.

## Summary.

The interaction of an antibody molecule with its ligand serves as the paradigm for immunological specificity, an essential concept in immunology. This is best understood by studying the binding of antibodies to antigens, which illustrates their tremendous power to discriminate between related antigens and their high affinity of binding to particular structures. The behavior of antibodies in serological assays shows that antibody molecules are highly diverse, symmetrically bivalent, and have both constant and variable structural features. How the immune system produces the millions of different antibody molecules found in serum while maintaining their overall structural identity that allows anti-immunoglobulin antibodies to detect any antibody molecule, is the main subject of Chapter 3. In Chapter 8, we will learn about the production of antibody by B cells and why the amount, specificity, isotype, and affinity of antibody molecules are important in humoral immunity; here, we have learned how these attributes can be measured in a wide variety of distinct assays, each giving its own type of information about the antibody response. Since antibodies can be raised to any structure, can bind it with high affinity and specificity, and can be made in unlimited amounts through monoclonal antibody production, they are particularly powerful tools of investigation. Many different techniques using antibodies have been devised and they have played a central role in both clinical medicine and biological research.

Related to Part III

# The study of lymphocytes.

The analysis of immunological specificity has focused largely on the antibody molecule as the most easily accessible agent of adaptive immunity. However, all adaptive immune responses are mediated by lymphocytes, so an understanding of immunology must be based on an understanding of lymphocyte behavior. To study and assay lymphocyte behavior the cells must be isolated and the distinct functional lymphocyte subpopulations identified and separated. This section emphasizes studies on T lymphocytes, as the only known effector function of B cells is to produce antibodies, the subject of the preceding part of this chapter.

## 2-16 Lymphocytes can be isolated from blood, bone marrow, lymphoid organs, epithelia, and sites of inflammation.

The first step in studying lymphocytes is to isolate them so that their behavior can be analyzed *in vitro*. Human lymphocytes can be isolated most readily from peripheral blood using density centrifugation over a step gradient consisting of a mixture of the carbohydrate polymer Ficoll™ and the dense iodine-containing compound metrizamide. This yields a population of mononuclear cells at the interface that has been depleted of red blood cells and most polymorphonuclear leukocytes or granulocytes (Fig. 2.26). The resulting population, called **peripheral blood mononuclear cells**, consists mainly of lymphocytes and monocytes. Although this population is readily accessible, it is not necessarily representative of the lymphoid system, as only recirculating lymphocytes can be isolated from blood. In experimental animals, and occasionally in humans, lymphocytes can be isolated from lymphoid organs, such as spleen, thymus, bone marrow, lymph nodes, or mucosal-associated lymphoid tissues, most commonly the palatine tonsils

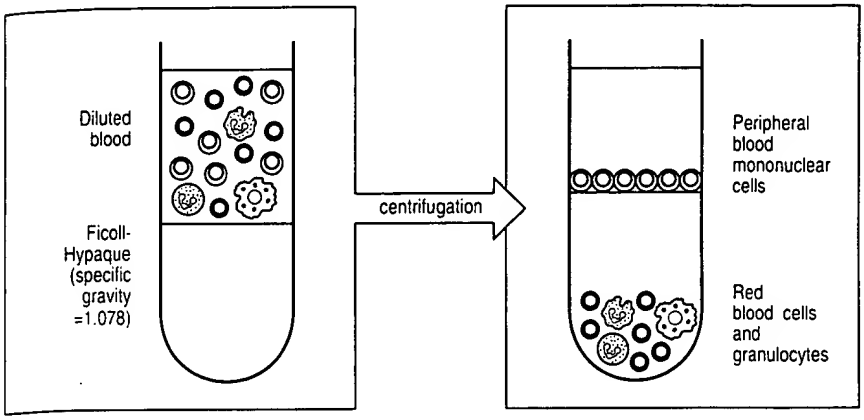



**Fig. 2.26 Peripheral blood mononuclear cells can be isolated from whole blood using Ficoll-Hypaque™ centrifugation.** Diluted anti-coagulated blood (left panel) is layered over Ficoll-Hypaque and centrifuged. Red blood cells and polymorphonuclear leukocytes or granulocytes are more dense and centrifuge through the Ficoll-Hypaque, while mononuclear cells consisting of lymphocytes together with some monocytes band over it and can be recovered at the interface (right panel).

in humans (see Fig. 1.6). A specialized population of lymphocytes resides in surface epithelia; these cells are isolated by fractionating the epithelial layer after its detachment from the basment membrane. Finally, in situations where local immune responses are prominent, lymphocytes can be isolated from the site of the response itself. For example, in order to study the autoimmune reaction that is thought to be responsible for rheumatoid arthritis, an inflammatory response in joints, lymphocytes are isolated from the fluid aspirated from the inflamed joint space.

Related to Part II

## 2-17 Lymphocyte populations can be purified and characterized by antibodies specific for cell-surface molecules.

Resting lymphocytes present a deceptively uniform appearance to the investigator, all being small round cells with a dense nucleus and little cytoplasm (see Fig. 1.5). However, these cells comprise many functional subpopulations, which are usually identified and distinguished from each other on the basis of their differential expression of cell-surface proteins, which can be detected using specific antibodies (Fig. 2.27). B and T lymphocytes, for example, are identified unambiguously and separated from each other by antibodies to the constant regions of the B- and T-cell antigen receptors. T cells are further subdivided on the basis of expression of the co-receptor proteins CD4 and CD8.

An immensely powerful tool for defining and enumerating lymphocytes is the **flow cytometer**, which detects and counts individual cells passing in a stream through a laser beam. A flow cytometer equipped to separate the identified cells is called a **fluorescence-activated cell sorter (FACS)**. These instruments are used to study the properties of cell subsets identified with monoclonal antibodies to cell-surface proteins. Individual cells within a mixed population are first tagged by treatment with specific monoclonal antibodies labeled with fluorescent dyes, or by specific antibodies followed by labeled anti-immunoglobulin. The mixture of labeled cells is then forced with a much larger volume of saline through a nozzle, creating a fine stream of liquid containing cells spaced singly at intervals. As each cell passes through a laser beam it scatters the laser light, and any dye molecules bound to the cell will be excited and will fluoresce. Sensitive photomultiplier tubes detect both the scattered light, which gives information on the size and granularity of the cell, and the fluorescence emissions, which give information on the binding of the labeled monoclonal antibodies and hence on the expression of cell-surface proteins by each cell (Fig. 2.28).

In the cell sorter, the signals passed back to the computer are used to generate an electric charge, which is passed from the nozzle through the liquid stream at the precise time the stream breaks up into droplets,

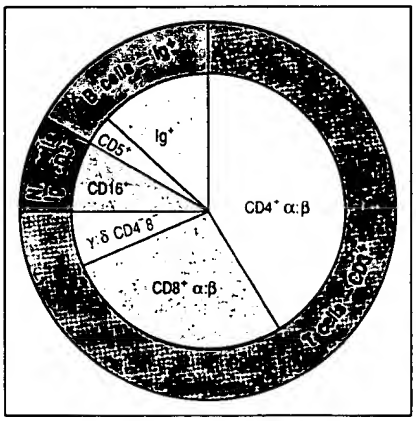



**Fig. 2.27 The distribution of lymphocyte subpopulations in human peripheral blood.** As shown on the outside, lymphocytes can be divided into T cells bearing T-cell receptors, detected with anti-CD3 antibodies, B cells bearing immunoglobulin receptors, detected with anti-immunoglobulin, and null cells including natural killer (NK) cells that label with neither. Further divisions of the T-cell and B-cell populations are shown inside. Using anti-CD4 and anti-CD8 antibodies, α:β T cells can be subdivided into two populations, while γ:δ T cells are identified with antibodies against the γ:δ T-cell receptor and mainly lack CD4 and CD8. A minority population of B cells express CD5 on their surface (see Section 5-14).

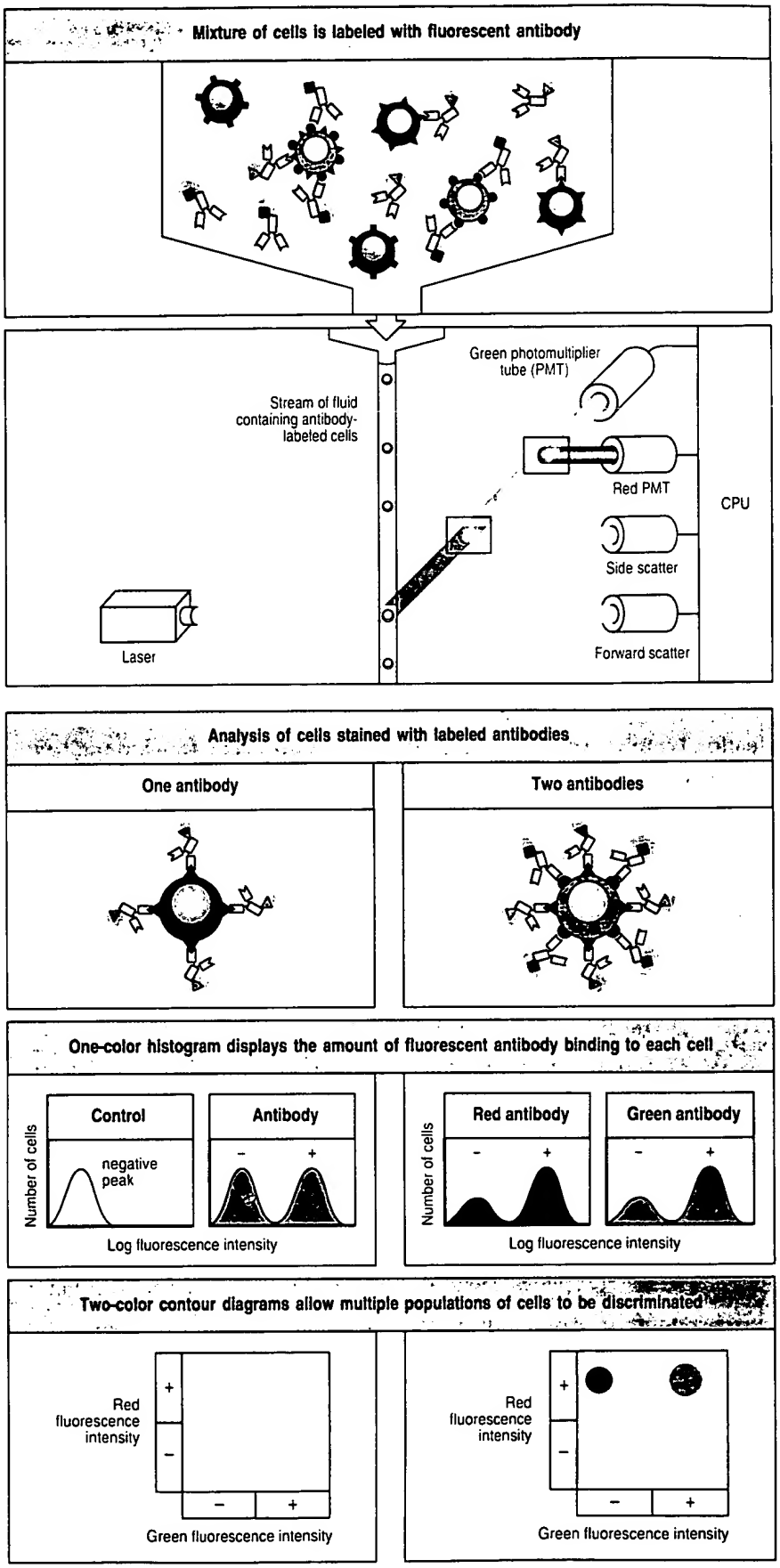



**Fig. 2.28 The FACS™ allows individual cells to be identified by their cell-surface antigens and to be sorted.** Cells to be analyzed by flow cytometry are labeled with fluorescent dyes (top panel). This can either be achieved directly using dye-coupled antibodies specific for cell-surface antigens (as shown here), or indirectly using a dye-coupled anti-immunoglobulin reagent to detect unlabeled cell-bound antibody. The cells are forced through a nozzle in a single-cell stream that passes through a laser beam (second panel). Photomultiplier tubes (PMTs) detect the scattering of light, which is a sign of cell size and granularity, and emissions from the different fluorescent dyes. This information is then analyzed using a computer. By examining many cells in this way, the number of cells with a specific set of characteristics can be counted and levels of expression of various molecules on these cells can be measured. As shown in the bottom left panels, use of a single antibody can indicate the percentage of a cell population bearing the molecule detected by that antibody, and the amount of that molecule expressed by each cell. This information can be presented as a one-color histogram (lower middle panels). Two-color contour profiles (bottom panels) are employed mainly when two or more antibodies are used. The use of two different dye-coupled specific antibodies (bottom right panels) can define four populations of cells: those expressing either molecule alone (red or green), those expressing both (orange), and those expressing neither (gray). The size and intensity of each circle indicates the numbers of cells with these characteristics. The same information is often portrayed in black and white by representing each cell with a dot.

Related to Part III

each containing no more than a single cell; droplets containing a charge can then be deflected from the main stream of droplets as they pass between plates of opposite charge, so that positively charged droplets are attracted to a negatively charged plate, and vice versa.

When cells are labeled with a single fluorescent antibody, the data from flow cytometers are usually displayed in the form of a histogram of fluorescence intensity versus cell numbers. If two or more antibodies are used, each coupled to different fluorescent dyes, then the data are more usually displayed in the form of a two-dimensional scatter diagram or as a contour diagram, where the fluorescence of one dye-labeled antibody is plotted against that of a second, with the result that a population of cells labeling with one antibody can be further subdivided by its labeling with the second antibody (see Fig. 2.28). By examining large numbers of cells, flow cytometry can give quantitative data on the percentage of cells bearing different molecules, such as surface immunoglobulin, which characterizes B cells, the T-cell receptor-associated molecules known as CD3, and the CD4 and CD8 co-receptor proteins that distinguish the major T-cell subsets. Likewise, FACS analysis has been instrumental in defining stages in the early development of B and T cells. FACS analysis has been applied to a broad range of problems in immunology; indeed, it played a vital role in the early identification of AIDS as a disease in which T cells bearing CD4 are depleted selectively (see Chapter 10).

Relate to Part II

Although the FACS is superb for isolating small numbers of cells in pure form, when large numbers of lymphocytes must be prepared quickly, mechanical means of separating cells are preferable. A powerful and accurate way of isolating lymphocyte populations is to expose them to paramagnetic beads coated with a monoclonal antibody that recognizes a distinguishing surface molecule. The tube containing the cells is then placed in a strong magnetic field, the cells attached to the beads are retained and cells lacking the surface molecule recognized by the monoclonal antibody can be decanted off, leaving behind only the bound cells that express that protein (Fig. 2.29). The bound cells are positively selected for expression of the determinant, while the unbound cells are

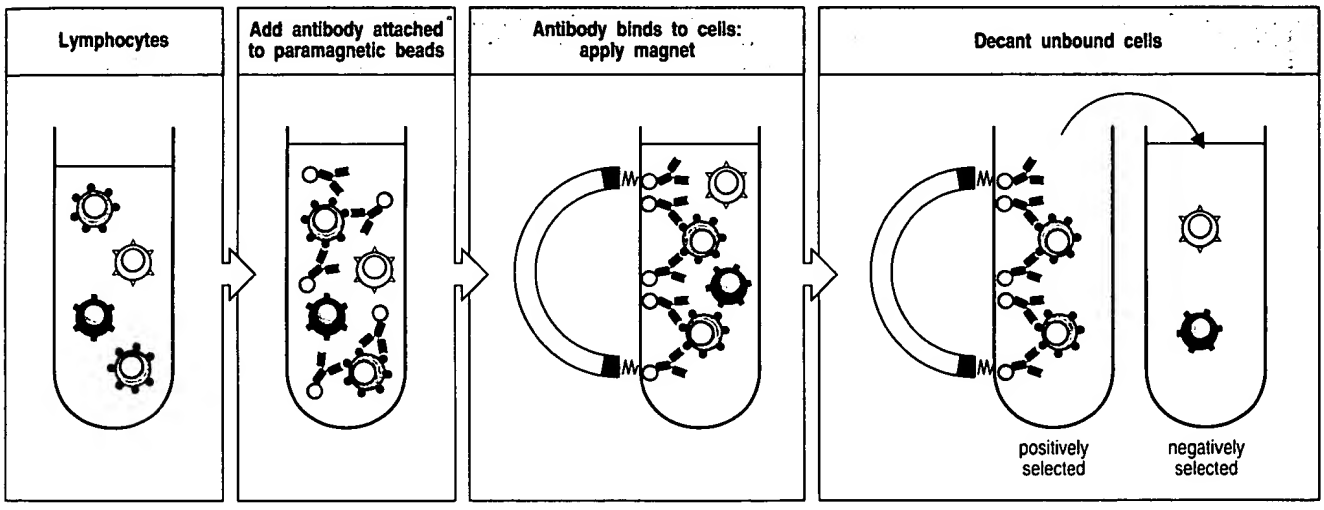



**Fig. 2.29 Lymph cyte subp pulati ns can b separated physically using antib dies c upled t paramagnetic beads.** Lymphocytes bearing a particular cell-surface molecule will bind to paramagnetic beads coupled with a mouse monoclonal antibody. They can be separated from cells that lack the molecule by attraction of the beads to a strong magnet. Unbound cells are decanted to yield a population that is said to be negatively selected for absence of the antigen recognized by the monoclonal antibody. Bound cells are recovered by warming or other treatments that disrupt antigen:antibody binding; they are said to be positively selected for presence of the antigen recognized by the antibody.

negatively selected for its absence. Cells have also been isolated by binding to antibody-coated plastic surfaces, a technique known as panning, or by killing cells bearing a particular molecule with specific antibody and complement (see Fig. 2.39). All these techniques can also be used as a pre-purification step prior to sorting out large numbers of highly purified populations by FACS.

The main conclusion reached from studies on isolated lymphocyte populations is that lymphocytes bearing particular combinations of cell-surface proteins represent distinct developmental stages that have particular functions, which suggested that these proteins must be involved directly in the function of the cell. For this reason, such surface molecules were originally called **differentiation antigens**. When groups of monoclonal antibodies were found to recognize the same differentiation antigen, they were said to define **clusters of differentiation**, abbreviated to **CD**, followed by an arbitrarily assigned number. This is the origin of the CD nomenclature for lymphocyte cell-surface antigens. The known CD antigens are listed in Appendix I.

## 2-18 Lymphocytes can be stimulated to grow by polyclonal mitogens or by specific antigen.

Related to Part IV

To function in adaptive immunity, rare antigen-specific lymphocytes must proliferate extensively before they differentiate into functional effector cells, in order to generate sufficient numbers of effector cells of a particular specificity. Thus, the analysis of induced lymphocyte proliferation is a central issue in their study. However, it is difficult to detect the proliferation of normal lymphocytes in response to specific antigen, because only a minute proportion of cells will be stimulated to divide. Enormous impetus was given to the field of lymphocyte culture by the finding that certain substances induce many or all lymphocytes of a given type to proliferate. These substances are referred to collectively as **polyclonal mitogens** because they induce mitosis in lymphocytes of many different specificities or clonal origins. T and B lymphocytes are stimulated by different polyclonal mitogens (Fig. 2.30). Polyclonal mitogens seem to trigger essentially the same growth response mechanisms as antigen. Lymphocytes normally exist as resting cells in the $G_0$ phase of the cell cycle. When stimulated with polyclonal mitogens, they rapidly enter the $G_1$ phase and progress through the cell cycle. In most studies, lymphocyte proliferation is most simply measured by the incorporation of $^3$H-thymidine into DNA. This assay is used clinically for assessing the ability of lymphocytes from patients with suspected immunodeficiencies to proliferate in response to a non-specific stimulus (see Section 2-31).

**Fig. 2.30 Polyclonal mitogens, many of plant origin, stimulate lymphocyte proliferation in tissue culture.** Many of these mitogens are used to test the ability of lymphocytes in human peripheral blood to proliferate.

| Mitogen | Abbreviation | Source | Responding cells |
|---|---|---|---|
| Phytohemagglutinin | PHA | *Phaseolus vulgaris* (red kidney beans) | T cells |
| Concanavalin A | ConA | *Canavalia ensiformis* (Jack bean) | T cells |
| Pokeweed mitogen | PWM | *Phytolacca americana* (pokeweed) | T and B cells |
| Lipopolysaccharide | LPS | *Escherichia coli* | B cells (mouse) |

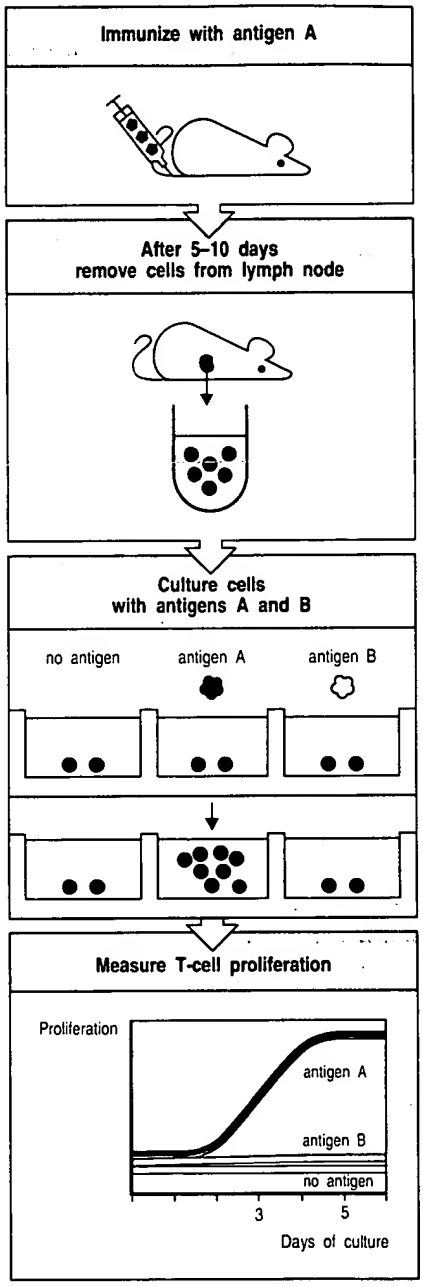



**Fig. 2.31 Antigen-specific T-cell proliferation is used frequently as an assay for T-cell responses.** T cells from mice or humans that have been immunized with an antigen (A) proliferate when they are exposed to antigen A and antigen-presenting cells but not to unrelated antigens to which they have not been immunized (antigen B). Proliferation can be measured by incorporation of $^{3}$H-thymidine into the DNA of actively dividing cells. Antigen-specific proliferation is a hallmark of specific CD4 T-cell immunity.

Once lymphocyte culture had been optimized using the proliferative response to polyclonal mitogens as an assay, it became possible to detect antigen-specific T-cell proliferation in culture by measuring $^{3}$H-thymidine uptake in response to an antigen to which the T-cell donor had been previously immunized (Fig. 2.31). This is the assay most commonly used for assessing T-cell responses after immunization, but it reveals little about the functional capabilities of the responding T cells. These must be ascertained by functional assays, as described in the next section.

## 2-19 T-cell effector functions can be measured in four ways—target-cell killing, macrophage activation, B-cell activation, or lymphokine production.

As we learned in Section 2-6, effector T cells are detected by their effects on target cells displaying antigen, or the secretion of specific cytokines that act on such target cells. Measuring these effector functions forms the basis for T-cell bioassays used to assess both T-cell specificity for antigen and T-cell effector functions.

Activated CD8 T cells generally kill any cells that display the specific peptide:MHC class I complex they recognize. Therefore CD8 T-cell function can be determined using the simplest and most rapid T-cell bioassay—the killing of a target cell by a cytotoxic T cell. This is usually detected in a $^{51}$Cr-release assay. Live cells will take up, but do not spontaneously release, radioactively labeled sodium chromate, $Na_2{}^{51}CrO_4$. When these labeled cells are killed, the radioactive chromate is released and its presence in the supernatant of mixtures of target cells and cytotoxic T cells can be measured (Fig. 2.32). In a similar assay, proliferating target cells such as tumor cells can be labeled with $^{3}$H-thymidine, which is incorporated into the replicating DNA. On attack by a cytotoxic T cell, the DNA of the target cells is rapidly fragmented and released into the supernatant, and one can measure either the release of these fragments or the retention of $^{3}$H-thymidine in chromosomal DNA. These assays provide a rapid, sensitive, and specific measure of the activity of cytotoxic T cells.

The fragmentation of the DNA of cells killed by cytotoxic T cells results from the induction of a process of programmed cell death, or apoptosis,

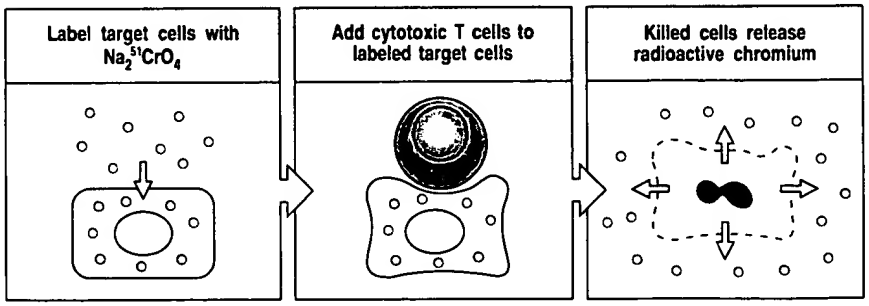



**Fig. 2.32 Cytotoxic T-cell activity is often assessed by chromium release from labeled target cells.** Target cells are labeled with radioactive chromium as $Na_2{}^{51}CrO_4$ and exposed to cytotoxic T cells. Cell destruction is measured by the release of radioactive chromium into the medium, detectable within 4 hours of mixing target cells with T cells.

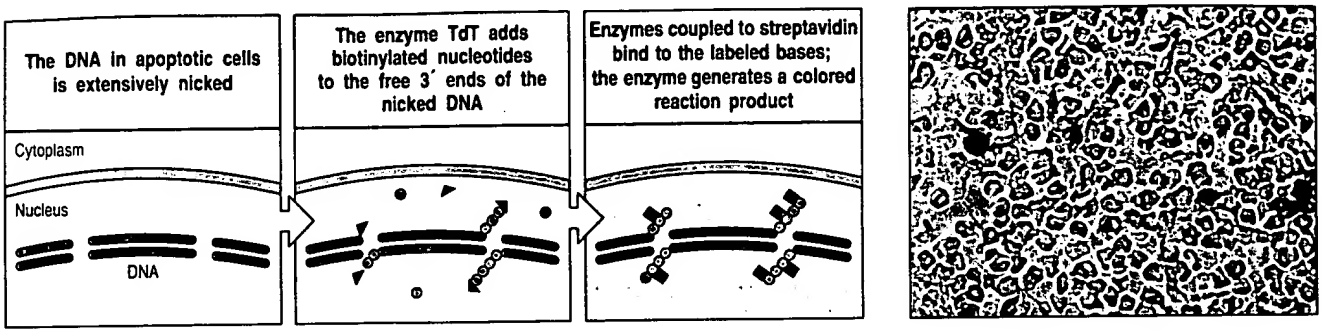



**Fig. 2.33 Fragmented DNA can be labeled by terminal deoxyribonucleotidyl transferase (TdT) to reveal apoptotic cells.** When cells undergo programmed cell death, or apoptosis, their DNA becomes fragmented (left panel). The enzyme TdT is able to add nucleotides to the ends of DNA fragments; in this assay, biotin-labeled dUTP is added (second panel). The biotinylated DNA can be detected using streptavidin, which binds to biotin, coupled to enzymes that convert a colorless substrate into a colored insoluble product (third panel). Cells stained in this way can be detected by light microscopy, as shown in the photograph of apoptotic cells (stained red, right panel). Photograph courtesy of R Budd and J Russell.

within the target cell. The characteristic cleavage of the DNA in cells undergoing programmed cell death has been used as the basis for an assay to identify apoptotic cells *in situ*. Nucleotides tagged with biotin are added to the cells, along with the enzyme terminal deoxyribonucleotidyl transferase (TdT) which can add the biotin-tagged nucleotides to the free 3′ ends of the DNA fragments produced by apoptosis. Reporter enzymes coupled to avidin or streptavidin will bind to the tagged nucleotides and can be used to identify apoptotic cells by converting a colorless substrate into a colored insoluble product (Fig. 2.33), in much the same way that such reagents are used in immunohistochemical staining (see Section 2-13). The labeled nucleotide most commonly used in this assay is dUTP, coupled to biotin. Hence the assay is often called the TdT-dependent dUTP–biotin nick end labeling, or **TUNEL assay**.

Related to Part IV.

CD4 T-cell functions usually involve the activation rather than the killing of cells bearing specific antigen, which for CD4 cells is a specific peptide: MHC class II complex. The activating effects of CD4 T cells on B cells or macrophages are mediated in large part by non-specific mediator proteins called cytokines, which are released by the T cell when it recognizes antigen (see Chapter 7). Thus, CD4 T-cell function is usually studied by measuring the type and amount of these released proteins. As different effector T cells release different amounts and types of cytokines, one can learn about the effector potential of that T cell by measuring the proteins it produces. Cytokines can be detected by their activity in biological assays of cell growth, where they serve either as growth factors or growth inhibitors, or more specifically by a modification of ELISA, known as a **capture** or **sandwich ELISA**. In this assay, the cytokine is characterized by its ability to bridge between two monoclonal antibodies reacting with different epitopes on the cytokine molecule (Fig. 2.34). Sandwich ELISA can also be carried

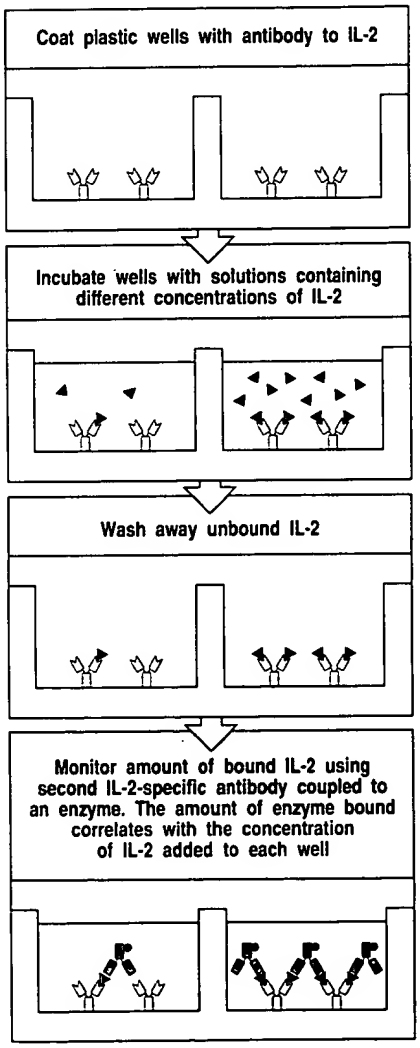



**Fig. 2.34 Measurement of interleukin-2 (IL-2) production by sandwich ELISA.** When T cells are activated with a mitogen or antigen they usually release the T-cell growth factor IL-2. The IL-2 can be measured by induction of growth of an IL-2 responsive indicator cell (not shown); however, assay for IL-2 by sandwich ELISA is far more accurate and specific. In this assay, one unlabeled anti-IL-2 antibody is attached to the plastic, and then the IL-2-containing fluid is added. After washing, bound IL-2 is detected by binding a second, labeled anti-IL-2 antibody directed at a different epitope. This assay is highly specific because antigens that cross-react with one antibody are very unlikely to cross-react with the other. It can detect and quantify IL-2 and many other cytokines with gr at sensitivity and precision.

out by placing the cells themselves on a surface coated with antibody to a cytokine. After a short incubation, the cytokine released by each cell is trapped on the antibody coat and the presence of cytokine-secreting cells can be revealed when the cells are washed off and a labeled second anti-cytokine antibody is added. The cytokine released by each cell makes a distinct spot in this assay, which is therefore known as an **ELISPOT assay**. ELISPOT can also be used to detect specific antibody secretion by B cells, in this case using antigen-coated surfaces to trap specific antibody, and labeled anti-immunoglobulin to detect the bound antibody. Sandwich ELISA avoids a major problem of cytokine bioassays, the ability of different cytokines to stimulate the same response in a bioassay. Bioassays must always be confirmed by inhibition of the response with monoclonal antibodies against the cytokine.

An alternative method is to identify cytokine mRNA, either in a cell population by reverse transcriptase-polymerase chain reaction (RT-PCR) or by *in situ* hybridization of single cells. Reverse transcriptase is an enzyme that is used by RNA viruses, like the human immunodeficiency virus that causes Acquired Immune Deficiency Syndrome (AIDS), to convert an RNA genome into a DNA copy, or cDNA. By harvesting mRNA from T cells stimulated with antigen, one can make cDNA copies, which are then amplified selectively using cytokine-specific primers by the polymerase chain reaction (see Fig. 2.44). The amount of product is proportional to its representation in the RNA in the responding cell. *In situ* hybridization uses labeled anti-sense RNA probes to hybridize with sense RNA in single cells, either isolated from culture or directly in tissue sections. This allows the number of cells making RNA encoding a particular cytokine to be determined.

## 2-20 Homogeneous T lymphocytes can be obtained as T-cell hybrids, cloned T-cell lines, or T-cell tumors.

Just as the analysis of antibody specificity and structure has been aided greatly by the development of hybridomas making monoclonal antibodies, the analysis of specificity and effector function in T cells has depended heavily on monoclonal populations of T lymphocytes. These can be obtained in three ways. First, as for hybridomas, normal T cells proliferating in response to specific antigen can be fused to malignant T-cell lymphoma lines to generate **T-cell hybrids**. The hybrids express the receptor of the normal T cell, but proliferate indefinitely owing to the cancerous state of the lymphoma parent. T-cell hybrids can be cloned to yield a population of cells all having the same T-cell receptor. These cells can be stimulated by specific antigen to release biologically active mediator molecules such as the T-cell growth factor interleukin-2, and the production of cytokines is used to assess the specificity of the T-cell hybrid.

T-cell hybrids are excellent tools for the analysis of T-cell specificity, as they grow readily in suspension culture. However, they cannot be used to analyze the regulation of specific T-cell proliferation in response to antigen because they are continually dividing. T-cell hybrids cannot be transplanted into an animal to test for function *in vivo* because they would give rise to tumors, and functional analysis of T-cell hybrids is also confounded by the fact that the malignant partner cell affects their behavior in functional assays. Therefore, the regulation of T-cell growth and the effector functions of T cells must be studied using cloned T-cell lines, derived from single T cells, whose growth is dependent on periodic restimulation with specific antigen and, frequently, the addition of T-cell growth factors (Fig. 2.35). Such cells are more tedious to grow but,

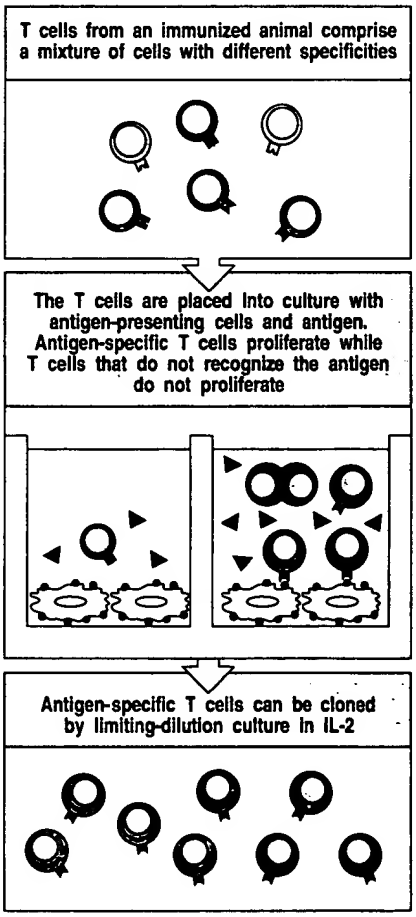



**Fig. 2.35 Production of cloned T-cell lines.** T cells from an immune donor, comprising a mixture of cells with different specificities, are activated with antigen and antigen-presenting cells. Single responding cells are cultured by limiting dilution (see Fig. 2.36) in the T-cell growth factor interleukin-2 (IL-2). From these single cells, cloned lines specific for antigen are identified and can be propagated by culture with antigen, antigen-presenting cells, and IL-2.

Relate to Part

because their growth depends on specific antigen recognition, they maintain antigen specificity, which is often lost in T-cell hybrids. Cloned T-cell lines can be used for studies of effector function both *in vitro* and *in vivo*. In addition, the proliferation of T cells, a critical aspect of clonal selection, can only be characterized in cloned T-cell lines where such growth is dependent on antigen recognition. Thus, both types of monoclonal T-cell line have valuable applications in experimental studies.

In studies of human T cells, T-cell clones have proven of greatest value because a suitable fusion partner for making T-cell hybrids has not been identified. However, a human T-cell lymphoma line, called Jurkat, has been characterized extensively because it secretes interleukin-2 when its antigen receptor is crosslinked with anti-receptor monoclonal antibodies. This simple assay system has yielded much information about signal transduction in T cells. One of the Jurkat cell line's most interesting features, shared with T-cell hybrids, is that it stops growing when its antigen receptor is crosslinked. This has allowed mutants lacking the receptor or having defects in signal transduction pathways to be selected simply by culturing the cells with anti-receptor antibody and selecting those that continue to grow. Thus, T-cell tumors, T-cell hybrids, and cloned T-cell lines all have valuable applications in experimental immunology.

## 2-21 Limiting dilution measures the frequency of lymphocytes specific for a particular antigen.

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Part IV

The response of a lymphocyte population is a measure of the overall response, but the frequency of specific lymphocytes able to respond to an antigen can only be determined by limiting dilution culture. This assay makes use of the Poisson distribution, a statistical function that describes how objects are distributed at random. For instance, when different numbers of T cells are distributed into a series of culture wells, some wells will receive no specific T cells, some will receive one specific T cell, some two and so on. The T cells are activated with specific antigen, antigen-presenting cells (APCs), and growth factors. After allowing several days for their growth and differentiation, the cells in each well are tested for a response to antigen, such as cytokine release or the ability to kill specific target cells. The logarithm of the proportion of wells in which there is no response is plotted against the linear number of cells initially added to the well. If cells of one type, typically antigen-specific T cells because of their rarity, are the only limiting factor for obtaining a response, then a straight line is obtained. From the Poisson distribution, it is known that there is, on average, one antigen-specific cell per well when the proportion of negative wells is 37%. Thus, the frequency of antigen-specific cells in the population equals the reciprocal of the number of cells added to the wells when 37% of the wells are negative. After priming, the frequency of specific cells goes up substantially, reflecting the antigen-driven proliferation of antigen-specific cells (Fig. 2.36). The limiting dilution assay can also be used to measure the frequency of B cells that can make antibody to a given antigen.

## Summary.

The cellular basis of adaptive immunity is the clonal selection of lymphocytes by antigen. Therefore, to study adaptive immune responses, one must isolate lymphocytes and characterize them. Lymphocytes can be divided into subpopulations using antibodies that detect cell-surface

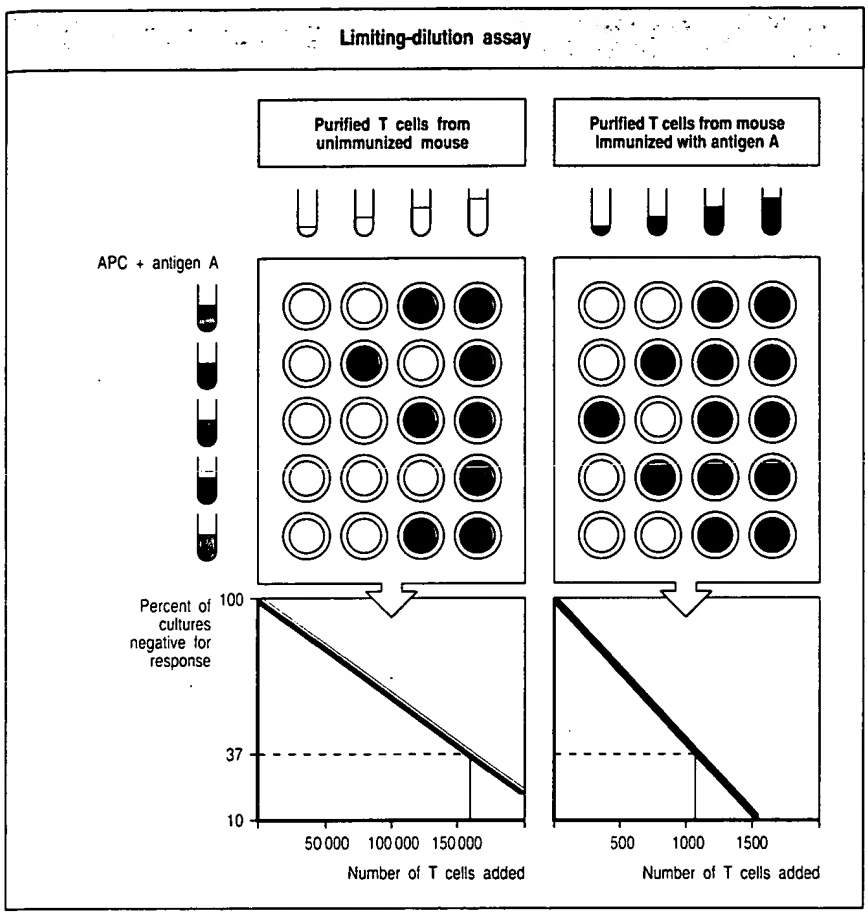


**Fig. 2.36 The frequency f specific lymph cytes can be determined using limiting dilution assay.** Varying numbers of lymphoid cells from normal or immunized mice are added to individual culture wells and stimulated with antigen and antigen-presenting cells (APCs) or polyclonal mitogen and added growth factors. After several days, the wells are tested for a specific response to antigen, such as cytotoxic killing of target cells. Each well that initially contained a specific T cell will make a response to its target, and from the Poisson distribution one can determine that when 37% of the cultures are negative, each well contained, on average, one specific T cell at the beginning of the culture. In the example shown, for the unimmunized mouse 37% of the wells are negative when 160 000 T cells have been added to each well; thus the frequency of antigen-specific T cells is 1 in 160 000. When the mouse is immunized, 37% of the wells are negative when only 1100 T cells have been added; hence the frequency of specific T cells after immunization is 1 in 1100, an increase in responsive cells of 150 fold.

Related to Part IV

molecules expressed selectively on cells of a given type. Subsets defined in this way also differ functionally, suggesting that the cell-surface molecules detected are important for the function of that cell. Antibodies to cell-surface antigens can be used to separate lymphocytes physically, using magnetic beads, or by fluorescence-activated cell sorting, which also allows quantitative analysis of cell subpopulations. The functional capabilities of these isolated populations can then be tested *in vitro* and *in vivo*. Individual T cells can also be cloned, either as T-cell hybridomas or as continuously growing lines of normal T cells, which are valuable for analyzing the specificity, function, and signaling properties of T cells.

## Immunogenetics: the major histocompatibility complex.

**Immunogenetics** includes the use of antibodies, and more recently of T cells, to detect genetic differences or polymorphisms within a population. The first polymorphic system to be studied by immunogenetics was the ABO blood group system defined by Karl Landsteiner, and virtually all blood typing is carried out using immunogenetic techniques (see Fig. 2.9). In no area has immunogenetics played such a vital

role as in the analysis of the highly polymorphic **major histocompatibility complex (MHC)** of genes (see Chapter 4). As we mentioned in Chapter 1, all T-cell responses involve the recognition of peptide fragments of antigen bound to cell-surface proteins encoded in the MHC (MHC molecules), making the analysis of the MHC a central concern of immunologists. The MHC in humans is the most polymorphic cluster of human genes known, and this polymorphism is of interest to immunologists because it affects antigen recognition by T cells (see Chapter 4). Its role in T-cell development (see Chapter 6), the rejection of tissue grafts (see Chapter 12), and susceptibility to many immunological disorders (see Chapters 11 and 12), provides a strong clinical impetus to the analysis of the MHC in humans. The MHC is also of great interest to students of evolution who study polymorphism in an effort to measure genetic history of particular genes or the role of natural selection in maintaining polymorphism. Here, we shall look at the techniques used to analyse MHC genetics and function. We will start by describing the graft rejection responses that are caused by MHC polymorphism and that first drew the attention of biologists to its existence.

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Part II

## 2-22 Tissues grafted between unrelated individuals are rejected.

The existence of a highly polymorphic MHC was first inferred from the rejection of grafted tissues. Blood transfusion had proved very successful once the genetics of the ABO blood group system had been deciphered, and this led to the idea that solid tissues could similarly be replaced by surgical transplantation. The first attempts to do so all failed: graft rejection was rapid and total, most grafts functioning for a brief period before becoming infiltrated with lymphocytes and dying (Fig. 2.37).

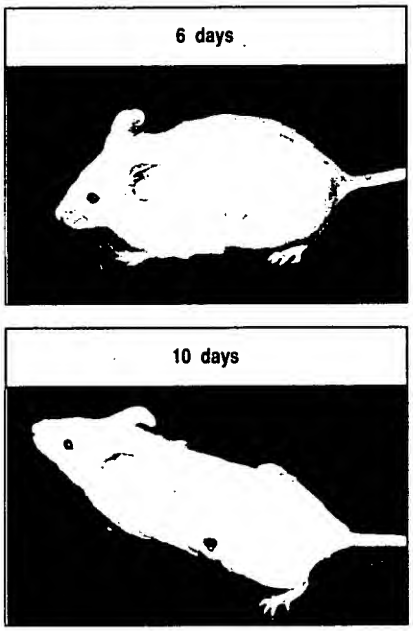


**Fig. 2.37 The immune response to a skin graft causes its rejection by the recipient.** Skin from mice of one genotype is grafted to a mouse of a different genotype. Six days after grafting, the skin is healthy (top panel). However, ten days after grafting, an adaptive immune response to the foreign antigens on the graft leads to its destruction (bottom panel).

Skin was the favored tissue for experimental transplants, as the graft could be examined directly on a regular basis. The genetic basis of graft rejection was first shown by the successful grafting of skin between individuals of an inbred mouse strain. Inbred mice have been specially bred so that they are homozygous at all loci. All members of an inbred strain carry identical alleles at each locus. These mice are equivalent to monozygotic human twins, and tissues can be grafted between monozygotic twins without rejection as well. Skin transplanted to inbred mice from members of other inbred strains or from other species was invariably rejected (Fig. 2.38). It was soon appreciated that the skin graft rejection was caused by an immune response against the grafted tissue. Graft rejection showed specific immunological memory, and immunodeficient mice did not reject grafts. However, the immunological and genetic basis of graft rejection remained to be worked out.

To analyze the genetic basis of this response, two inbred strains that mutually reject skin grafts were bred with each other, so that their F1 hybrid offspring had one set of alleles from each parental strain. When grafts were made between the F1 hybrid offspring and the parental strains, the F1 hybrid accepted skin from both parental strains, while the skin of the F1 hybrid was rejected by both parents. This showed that all the antigens involved in transplant rejection are expressed in the F1 hybrid mouse, as both parents reject its skin; and that its immune system is tolerant of all such antigens, as it accepts grafts from each parental strain. What was of particular interest was that half the offspring of F1 mice backcrossed to one parental strain rapidly rejected grafts of the other parental strain, starting about 8 days after grafting, indicating that a single genetic locus must control rapid graft rejection. This locus was later found to be composed of a cluster of related genes, and was

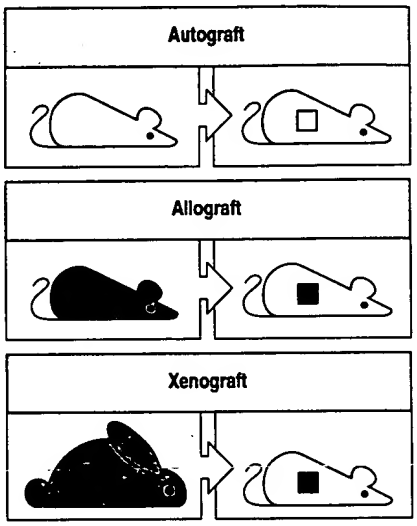


**Fig. 2.38 Th termin l gy f transplantati n.** Grafts from one site to another on the same individual are termed autologous grafts or autografts and are accepted. Grafts between genetically identical individuals, including those between members of the same inbred strain or between identical twins, are called syngeneic grafts, behave as autografts, and are also accepted. Grafts between genetically non-identical or allogeneic members of the same species are called allografts and are rejected. Grafts between the members of different species are called xenografts and are rejected.

termed the major histocompatibility complex (MHC) because it is the major determinant of graft survival. The genetic locus determining rapid graft rejection was found to segregate precisely with an antigen detected by an antibody that reacted with mouse red blood cells. This antigen was known as antigen-2, and the locus it defined was named histocompatibility-2 or **H-2**, the genetic designation for the mouse MHC. Subsequently, it was discovered that grafts between mice identical at H-2 were also rejected, although rejection generally occurred longer after grafting. These mice were shown to differ at other genetic loci encoding **minor histocompatibility (H) antigens**.

As genetic matching at H-2 led to prolonged survival of tissue grafts, it was believed that identification of a similar genetic complex might allow tissue grafting in humans. The human MHC was studied first using antibody reactions with white blood cells (Fig. 2.39) and is now called the **human leukocyte antigen**, or **HLA**, system. Unfortunately, the remarkable polymorphism of HLA in the outbred human population makes the identification of HLA-identical individuals enormously difficult. Furthermore, the presence of minor H antigens in humans means that it is only possible to to achieve the perfect matching that is available in genetically identical members of an inbred mouse strain when the human donor and recipient are monozygotic twins (see Chapter 12).

Related to Part II

## 2-23 MHC congenic, recombinant, and mutant inbred mouse strains are essential tools for analyzing MHC function.

The MHC is a complex of many closely linked genes, and most of the genes that encode MHC molecules that present antigens to T cells are highly polymorphic in both mice and humans. To study the function of the MHC and the effect of MHC polymorphism on immune responses, animals are needed that differ genetically only at the MHC. To produce such animals, George Snell took advantage of the observation that a tumor transplanted from one mouse would grow progressively in another mouse only if the recipient mouse carried the same MHC genes expressed by the tumor. Under these conditions, the recipient is tolerant to the tumor's MHC antigens and fails to reject it, so the tumor grows and kills the recipient mouse. One tumor used by Snell arose in mice of strain A and would therefore grow in and kill any strain A mouse it was injected into, so Snell crossed strain A mice (MHC genotype H-$2^a$) with mice of a different MHC genotype, such as strain C57BL/6 (MHC genotype H-$2^b$), to generate F1 hybrid mice (H-$2^{axb}$), which were then intercrossed to generate F2 mice. The F2 generation was injected with the tumor,

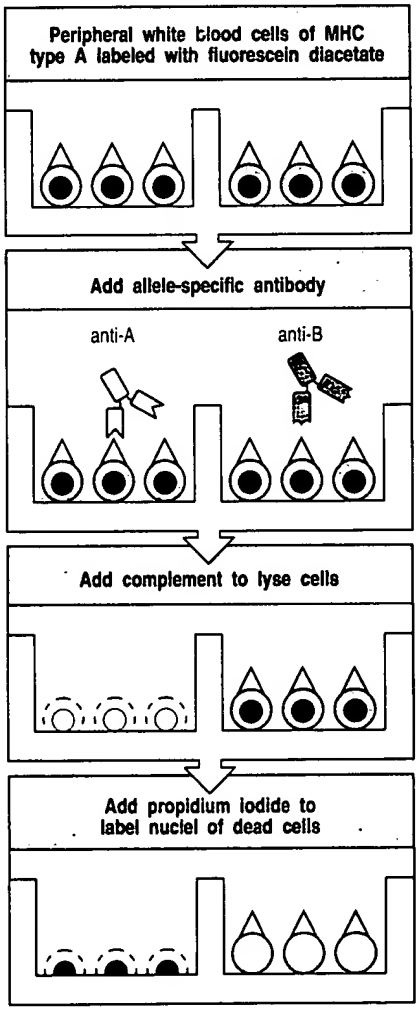


**Fig. 2.39 The microcytotoxicity assay is used in histoc mpatibility testing.** Leukocytes labeled with the vital dye fluorescein diacetate, which stains viable cells green, are exposed to antibody specific for allelic variants of MHC proteins and then exposed to complement. If the antibody reacts with the MHC proteins on a cell, this activates the complement so that the cell is killed, as seen by loss of green cells and the appearance of cells labeled by uptake of the dye propidium iodide (red), which only enters dead cells.

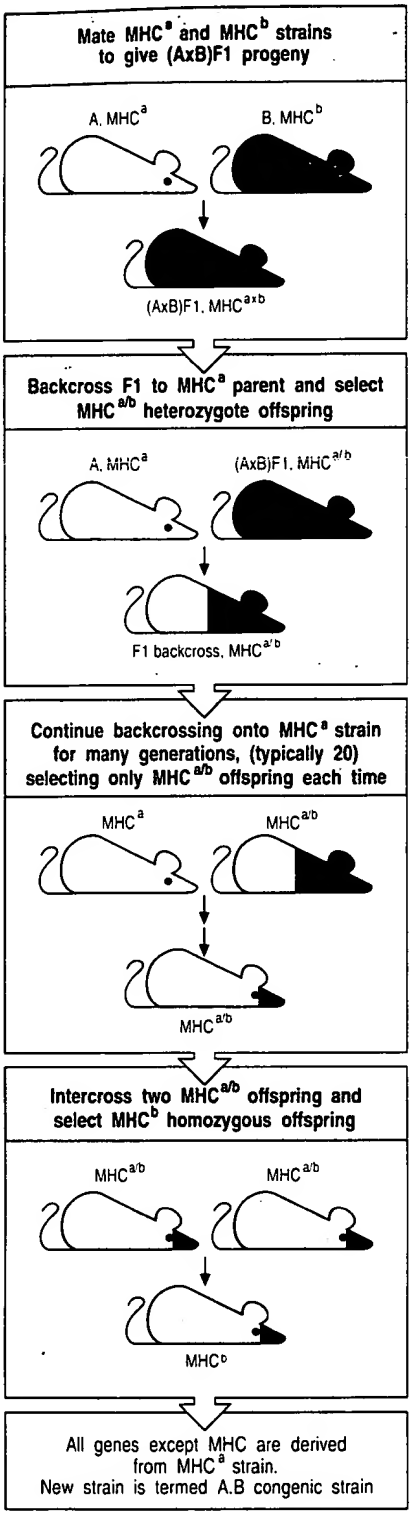


**Fig. 2.40 The production of MHC congenic mouse strains.** Mice of two strains, strain A with MHC genotype a (yellow) and strain B with MHC genotype b (blue) are crossed, and their F1 hybrid offspring, which are heterozygous at all loci including MHC$^{axb}$ (green), are backcrossed to parental strain A. The F1 backcross progeny, which are homozygous A at 50% of their loci (yellow) are selected for expression of the other parental MHC$^b$ and those that are MHC$^{axb}$ are backcrossed again to strain A. This continues for 10–30 backcross generations, after which the mice are homozygous A at virtually all loci except MHC, where they are a/b (green). These mice are intercrossed and selected for homozygosity at the MHC for alleles of donor origin (MHC$^b$) (blue). Virtually all the rest of the genome is derived from strain A to which the MHC genotype MHC$^b$ has been introgressively backcrossed. These mice are strain A co-isogeneic or congenic for MHC$^b$, and are designated A.B. They can be used to determine if genetic traits that differ between strain A and strain B map to the MHC.

and the one mouse in four that was homozygous H-2$^b$ survived because it was intolerant of H-2$^a$. These mice were then backcrossed to strain A and the progeny again intercrossed and challenged with the tumor. After 10 backcross/intercross generations, Snell obtained a mouse that was genetically 99% strain A but was homozygous for the MHC of the other parent in the initial cross, in this case H-2$^b$. These are called **congenic resistant** (to the strain A tumor) or **MHC congenic** mice, and are designated A.B, where B denotes the strain designation of the parent donating the MHC genes, in this case B6. This experiment illustrates not only MHC genetics and function but also that the immune system can combat tumors provided that they express recognizable antigens. We shall return to tumor immunity as a potential therapy for naturally occurring cancers in Chapter 13.

Congenic resistant mice have been crucial to our understanding of the role of the MHC in immunobiology. Fortunately there are now easier ways of producing them. For instance, antibodies to allelic variants of MHC proteins can be used to identify offspring that inherit the new MHC genes being bred onto strain A. One can therefore simply select the mice at each backcross generation that carry these genes, speeding the derivation of the new strains by avoiding the intercross generation (Fig. 2.40). During this process, it is also possible to detect mice that have undergone recombination within the MHC, such that only some MHC alleles are inherited from the donor parent. These are called **MHC recombinant strains**. These intra-MHC recombinant mice allow one to map a particular phenotype to a particular region of the MHC, further refining the genetic map. Although the molecular structure of the MHC is now well defined, *in vivo* analysis of MHC function still depends largely on such recombinant and congenic mice. For example, one can map traits to a single locus in the MHC using **MHC mutant** strains of mice (inbred mice differing only at a single MHC locus), or by using transgenic or gene knock-out mice, as we shall see in Section 2-37.

## 2-24 T lymphocytes respond strongly to MHC polymorphisms.

Graft rejection is carried out by T cells that recognize foreign MHC molecules and destroy the graft. Although this process can be studied *in vivo*, experimental analysis, especially in humans, required the development of *in vitro* correlates of graft rejection. One such assay is provided by the **mixed lymphocyte reaction (MLR)**, in which T cells are co-cultured with so-called stimulator cells (Fig. 2.41). These are usually irradiated peripheral blood lymphocytes, together with some antigen-presenting cells from an unrelated individual who is therefore likely to

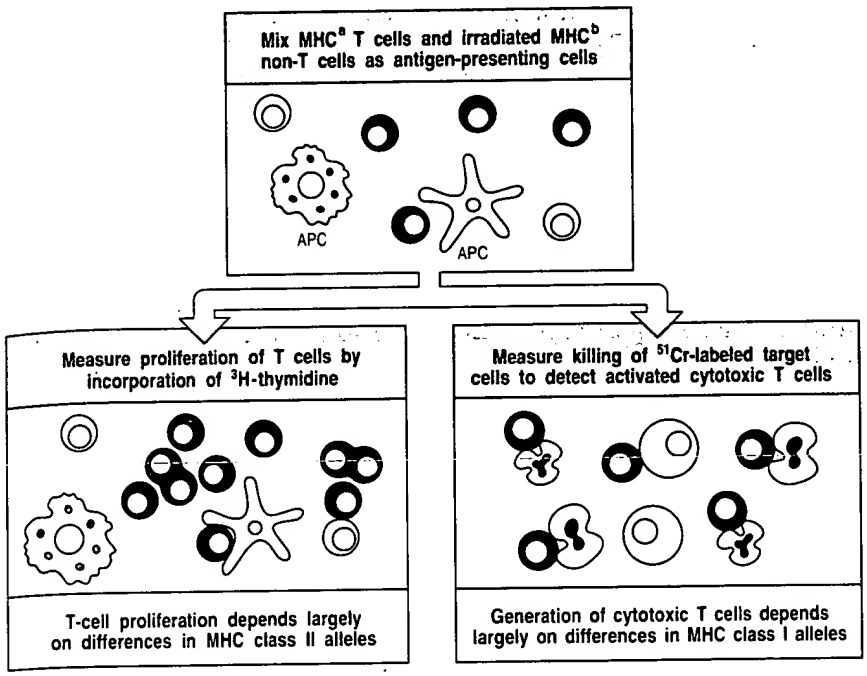


**Fig. 2.41 The mixed lymphocyte reaction (MLR) can be used for detecting histocompatibility.** Lymphocytes from two people are isolated from the peripheral blood. Cells of one person serve as the responders (blue), while the cells of the other donor (yellow) are used as stimulator cells. The non-T cells, which will include antigen-presenting cells, are irradiated or treated with the antibiotic mitomycin C before culture to block DNA synthesis and cell division. Between three to seven days after mixing the cells, the cultures are assessed for T-cell proliferation by measuring the uptake of $^3$H-thymidine, which is mainly the result of CD4 T cells recognizing differences in MHC class II genes, and for the generation of cytotoxic CD8 T cells that respond to differences MHC class I genes by the chromium-release assay on labeled target cells.

Related to Part II

be MHC disparate. The T cells are stimulated to proliferate and differentiate into effector cells; the irradiation of the stimulator cells prevents them from responding back. The T cells respond because they recognize MHC molecules on the stimulating cells that are different from their own MHC molecules.

The strong T-cell proliferative response in mixed lymphocyte culture reflects the high frequency of responding T cells. By limiting dilution assay (see Fig. 2.36), normal lymphocytes that respond to MHC differences are estimated to be present at between 1 cell in 500 and 1 cell in 20. The reason for this will be discussed in Chapters 4 and 6. The proliferative response is largely the result of CD4 T-cell recognition of MHC class II polymorphisms (see Fig. 2.41; lower left panel), while the cytotoxic T cells that result are predominantly CD8 T cells recognizing MHC class I polymorphisms (see Fig. 2.41, lower right panel). This *in vitro* correlate of graft rejection is very useful in screening for histoincompatibility between potential donors and recipients, as it is extremely sensitive and more closely related to the actual graft rejection response than is the microcytotoxicity assay carried out with antibodies (see Fig. 2.39). Unfortunately, it is also more cumbersome and expensive to carry out, and takes several days to produce an answer.

## 2-25 Antibodies to MHC molecules inhibit T-cell responses.

MHC polymorphism not only accounts for graft rejection but also has profound effects on antigen recognition by T cells, as we shall learn in Chapter 4. As the MHC encodes several different proteins that present antigen to T cells, it is often difficult using genetics alone to determine which MHC molecule a T cell is recognizing.

An alternative approach is to use monoclonal antibodies that bind to a given MHC molecule and prevent its recognition by the T-cell receptor (Fig. 2.42). Thus, proliferative responses of CD4 T cells in mixed lymphocyte reactions are largely inhibited by antibodies to MHC class II molecules, while antibodies to MHC class I molecules

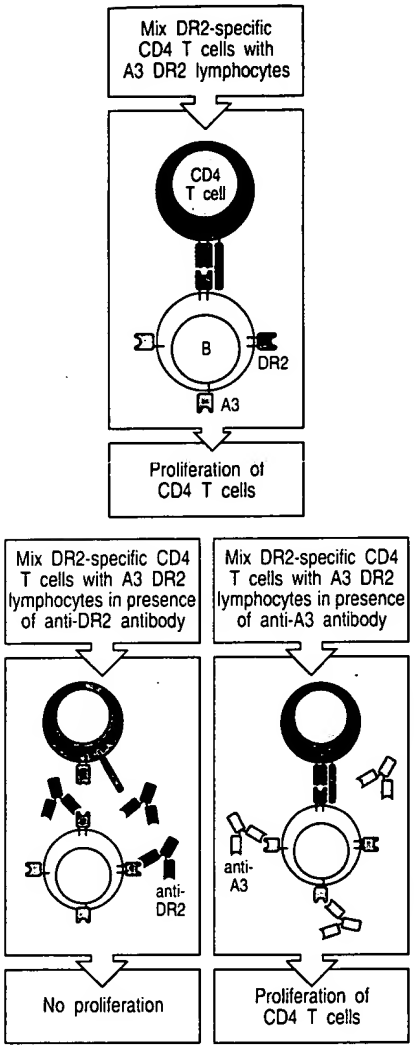


**Fig. 2.42 Antibody to specific MHC molecules can inhibit the mixed lymphocyte reaction.** A CD4 T-cell response to the MHC Class II molecule HLA-DR2 is inhibited by anti-HLA-DR2 antibodies, which compete with the T-cell receptor for binding to HLA-DR2. However, antibodies to the MHC Class I molecule HLA-A3 do not inhibit the response of CD4 T cells to HLA-DR2, even though they bind to the stimulator cell surface. This shows that antibodies that bind the stimulator cells inhibit specific recognition events and are not interfering with the response non-specifically.

block the cytolytic response of CD8 T cells in the same culture. Similarly, antibodies to MHC molecules can be used to define which MHC molecule is presenting peptides to an antigen-specific T cell. Antibodies to other structures crucial for cell interactions likewise inhibit these responses, so the use of monoclonal antibodies to inhibit T-cell responses has played a critical role in our understanding of these processes.

## 2-26 Antibodies to MHC molecules can be used to define the MHC genotype.

Although T cells can be used to detect MHC variability, routine genetic typing for MHC uses antibodies that distinguish between the numerous different allelic variants of any MHC molecule. Most of our information about MHC genetics has been, and continues to be, generated in this way. Antibody typing has defined multiple gene loci within the MHC, each with a large number of alleles in the two species studied most extensively, humans and mice. These loci comprise a tightly linked complex of genes on chromosome 6 in humans and chromosome 17 in mice. As the MHC genes are close together on the chromosome, genetic recombination rarely occurs within the MHC, and most individuals will inherit an intact set of parental alleles from each parent; such a set of linked genes is referred to as a **haplotype**, the MHC genes found in one haploid genome. The tight genetic linkage of MHC genes can be documented readily by genotyping family members (Fig. 2.43).

Population and family studies have revealed a striking association of HLA genotype with the incidence of a number of immune-mediated diseases. For some of these diseases, susceptibility was linked to a particular MHC class I genotype, but the majority were affected more strongly by genotype at the MHC class II locus. This linkage has stimulated the analysis of MHC polymorphism because the accuracy of disease-association analyses depends on how precisely the HLA genotypes of unrelated people can be determined and compared. Fortunately, the molecular analysis of MHC alleles is now possible, as we shall see in the next section.

## 2-27 Accurate MHC genotyping requires direct analysis of DNA sequence.

Although serological analysis is the main method for HLA genotyping humans, the reagents used are not specific enough to determine the precise structural identity of MHC molecules in unrelated individuals, who may have inherited closely related but distinct genes. Thus, while

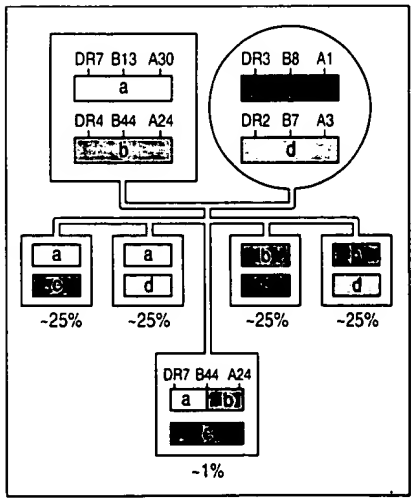


**Fig. 2.43 The inheritance of MHC haplotypes in families.** Each parent contributes genes from one of their two haplotypes, usually designated as a, b, c, and d. Most offspring inherit a complete MHC haplotype from each parent and can be designated simply a/c, a/d, b/c, or b/d; recombination within MHC haplotypes occurs at a frequency of only 1–2% (bottom panel).

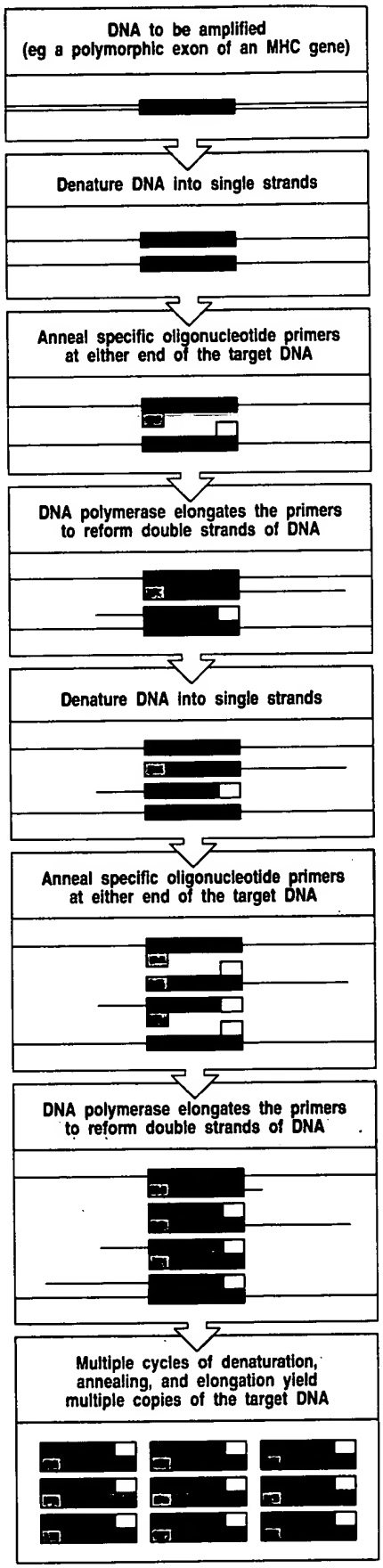


**Fig. 2.44 The p lymerase chain reaction.** To amplify a specific region of genomic DNA, such as a polymorphic exon of an MHC gene, synthetic oligonucleotide primers complementary to the DNA sequence flanking that region are made. The genomic DNA is denatured in the presence of an excess of these two oligonucleotide primers so that after reannealing, the primers have bound their complementary sequence in genomic DNA. The DNA polymerase Taq from the bacterium *Thermus aquaticus*, which is stable at the high temperatures used to denature DNA between replication cycles, elongates the primer using the genomic DNA between the two primers as a template. The replicated DNA is separated into single strands by heating and then the mixture is cooled so that a new cycle of primer annealing and replication can commence. The first extension products are random in length but as the reaction continues, the products that are delimited by the primers accumulate and hence are of the same length.

serological typing can accurately predict genotype in family members for shared MHC alleles, the identity of MHC alleles in unrelated individuals can be established only by direct structural analysis of their genes. This is now most conveniently performed using the **polymerase chain reaction** (**PCR**), a rapid method of selectively replicating a particular stretch of genomic DNA *in vitro* (Fig. 2.44). Once enough DNA is produced from the gene being amplified, it can be sequenced. Sequence analysis has shown that serologically identical alleles actually comprise several closely related alleles. There are now many known sequence variants of most serologically defined MHC alleles, which are quite similar to each other but differ by one or a few amino acids, whereas the proteins of different serologically determined alleles differ from one another by many amino acids.

Once the DNA sequence of a given allele has been determined, oligonucleotide probes can be constructed from the regions where differences from other alleles occur, and these differences can be detected by direct hybridization of the probe to PCR-amplified genomic DNA. This provides a rapid, cheap, and sensitive means of defining MHC gene structure. The application of this technique to MHC genetics has allowed the accurate determination of associations between MHC genes and several immunological diseases, and should help ultimately in determining the mechanism by which certain MHC alleles confer genetic susceptibility to particular diseases.

## Summary.

The cell-surface glycoproteins encoded by the MHC play a central role in immunology. As we learned in Chapter 1, their main function is to deliver peptide fragments of antigen to the cell surface where the peptide: MHC complex can be recognized by T cells. However, the MHC was originally discovered as the major genetic barrier to transplantation, because the strong response of T cells to foreign MHC molecules causes graft rejection. T-cell recognition of peptide antigens is also influenced profoundly by MHC polymorphism. Thus, the analysis of this polymorphism is essential in immunology. It is also important in clinical medicine, not only as a way to match graft donors with recipients but also for studying the role of MHC genotype in determining susceptibility to many human allergic and autoimmune diseases. Such studies require reliable genotyping of the MHC in humans, which is best carried out using DNA analysis. Experimental studies of MHC polymorphism are facilitated greatly by specialized MHC congenic, recombinant and mutant strains of mice. These clinical and experimental tools are essential for examining the impact of MHC polymorphism on immunity and immunological diseases.

Related to Part II

## Analyzing immune responses in intact or manipulated organisms.

The ultimate goal of immunology is to understand the immune response *in vivo* and to control it. To do so, techniques to study immunity in live animals and in human patients are essential. The following sections describe how immunity is measured and characterized in the intact organism, be it a mouse or a human being. From these observations much is learned about the functioning of the intact immune system. The cellular and molecular basis for these observed functions is the subject of much of this book. Experimental animals, and in particular inbred mice, can also be manipulated by various means for the purposes of studying immune functions. This can be achieved by transfering lymphocytes or antibodies, or by altering the genome, either by inserting new genes to create transgenic animals, or deleting genes using gene knock-out techniques.

### 2-28 Protective immunity can be assessed by challenge with infectious agents.

An adaptive immune response against a pathogen often confers long-lasting immunity against infection with that pathogen; successful vaccination achieves the same end. The very first experiment in immunology, Jenner's successful vaccination against smallpox, is still the model for assessing the presence of such protective immunity. The assessment of protective immunity conferred by vaccination has three essential steps. First, an immune response is elicited by immunization with a candidate vaccine. Second, the immunized individuals, along with unimmunized controls, are challenged with the infectious agent. Finally, the prevalence and severity of infection in the immunized individual is compared with the course of the disease in the unimmunized controls (Fig. 2.45). For obvious reasons, such experiments are usually carried out first in animals, if a suitable animal model for the infection exists. However, eventually a trial must be carried out in humans. In this case, the infectious challenge is usually provided naturally by carrying out the trial in a region where the disease is prevalent. The efficacy of the vaccine is determined by assessing the prevalence and severity of new infections in the immunized and control populations. Such studies necessarily give less precise results than a direct experiment but, for most diseases, they are the only way of assessing a vaccine's ability to induce protective immunity in humans.

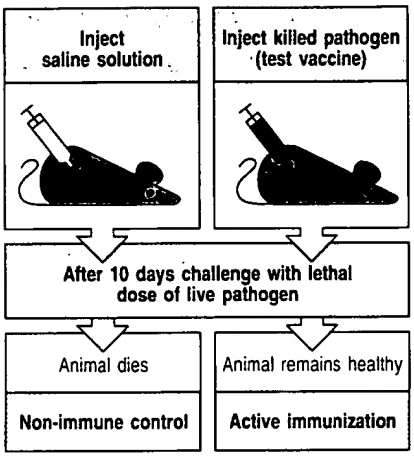


**Fig. 2.45 *In vivo* assay for the presence and nature of protective immunity after vaccination in animals.** Mice are injected with the test vaccine or a control such as saline solution. Different groups are then challenged with lethal or pathogenic doses of the test pathogen or with an unrelated pathogen as a specificity control (not shown). Unimmunized animals die or become severely infected. Successful vaccination is seen as specific protection of immunized mice against infection with the test pathogen. This is called active immunity and the process is called active immunization.

### 2-29 Immunity can be transferred by antibodies or by lymphocytes.

The tests described in the previous section show that protective immunity has been established but cannot show whether it involves humoral immunity, cell-mediated immunity, or both. When these studies are carried out in immunized or previously infected inbred mice, the nature of protective immunity can be determined by transferring serum or lymphoid cells from an immunized donor to an unimmunized syngeneic recipient (that is, a genetically identical animal of the same inbred strain) (Fig. 2.46). If protection against infection can be conferred by the transfer of serum, the immunity is provided by circulating antibodies and is called **humoral immunity**. Transfer of immunity by antiserum or purified antibodies provides immediate protection against many pathogens and against toxins such as those of tetanus and snake venom. However,

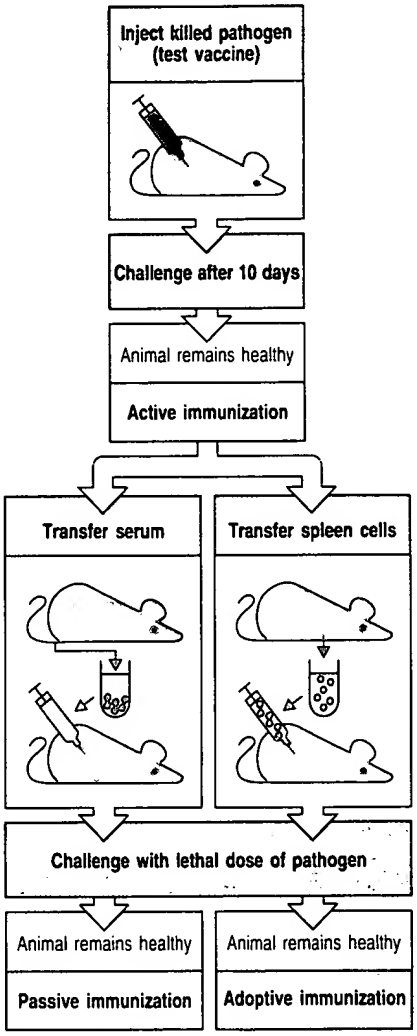


**Fig. 2.46 Immunity can b transferred by antib di s r by lymphocytes.** Successful vaccination leads to a long-lived state of protection against the specific immunizing pathogen. If this immune protection can be transferred to a normal syngeneic recipient with serum from an immune donor, then immunity is mediated by antibodies; such immunity is called humoral immunity and the process is called passive immunization. If immunity can only be transferred by infusing lymphoid cells from the immune donor into a normal syngeneic recipient, then the immunity is called cell-mediated immunity and the transfer process is called adoptive transfer or adoptive immunization. Passive immunity is short-lived, as antibody is eventually catabolized, but adoptively transferred immunity is mediated by immune cells, which can survive and provide longer-lasting immunity.

although protection is immediate, it is temporary, lasting only so long as the transferred antibodies remain active in the recipient's body. This type of transfer is therefore called **passive immunization**. Only **active immunization** with antigen can provide lasting immunity.

Protection against many diseases cannot be transferred by serum but can be transferred by lymphoid cells from immunized donors. The transfer of lymphoid cells from an immune donor to a normal syngeneic recipient is called **adoptive transfer** or **adoptive immunization**, and the immunity transferred is called **adoptive immunity**. Immunity that can be transferred only with lymphoid cells is called cell-mediated immunity. Such cell transfers must be between genetically identical donors and recipients, such as members of the same inbred strain of mouse, so that the donor lymphocytes are not rejected by the recipient and do not attack the recipient's tissues. Adoptive transfer of immunity is not used clinically in humans except in experimental approaches to cancer therapy or as an adjunct to bone marrow transplantation.

## 2-30 Local responses to antigen can indicate the presence of active immunity.

Active immunity is often studied *in vivo*, especially in humans, by injecting antigens locally in the skin. If a reaction appears, this indicates the presence of antibodies or immune lymphocytes that are specific for that antigen; the **tuberculin test** is an example of this. When people have had tuberculosis they develop cell-mediated immunity that can be detected as a local response when their skin is injected with a small amount of tuberculin, an extract of *Mycobacterium tuberculosis*, the pathogen that causes tuberculosis. The response typically appears a day or two after the injection and consists of a raised, red, and hard (or indurated) area in the skin, which then disappears as the antigen is degraded.

The immune system can also make less desirable responses, such as the hypersensitivity reactions responsible for allergies (see Chapter 11). Local intracutaneous injections of minute doses of the antigens that cause allergies are used to determine what antigen triggers a patient's allergic reactions. Local responses that happen in the first few minutes after antigen injection in immune recipients are called **immediate hypersensitivity reactions**, and they can be of several forms, one of which is the wheal-and-flare response described in Chapter 11. Immediate hypersensitivity reactions are mediated by specific antibodies of the IgE class formed as a result of earlier exposures to the antigen. Responses that take hours to days to develop, such as the tuberculin test, are referred to as **delayed-type hypersensitivity** responses and are caused by pre-existing immune T cells. This latter type of response was observed by Jenner when he tested vaccinated individuals with a local injection of vaccinia virus.

Related to Part V

These tests work because the local deposit of antigen remains concentrated in the initial site of injection, eliciting responses in local tissues. They do not cause generalized reactions if sufficiently small doses of antigen are used. However, local tests carry a risk of systemic allergic reactions, and they should be used with caution in people with a history of hypersensitivity.

## 2-31 The assessment of immune responses and immunological competence in humans.

The methods used for testing immune function in humans are necessarily more limited than those used in experimental animals, but many different tests are available, some of which have been mentioned already. They fall into several groups depending on the reason the patient is being studied.

Assessment of protective immunity in humans generally relies on tests conducted *in vitro*. To assess humoral immunity, specific antibody levels in the patient's serum are assayed using the test microorganism or a purified microbial product as antigen. To test for humoral immunity against viruses, antibody production is often measured by the ability of serum to neutralize the infectivity of live virus for tissue culture cells. In addition to providing information about protective immunity, the presence of antibody to a particular pathogen indicates that the patient has been exposed to it, making such tests of crucial importance in epidemiology. At present, testing for antibody to HIV is the main screening test for infection, critical both for the patient and in blood banking, where blood from infected donors must be excluded from the supply. Essentially similar tests are used in investigating allergy, where allergens are used as the antigens in tests for specific IgE antibody by ELISA or radioimmunoassay (see Section 2-7), which may be used to confirm the results of skin tests.

Related to Part V

Cell-mediated immunity to infectious agents can be tested either by skin test with extracts of the pathogen, as in the tuberculin test (see Section 2-30), or by the ability of the pathogen or an extract from it to stimulate T-cell proliferative responses *in vitro* (Section 2-18). These tests provide information about the exposure of the patient to the disease and also about their ability to mount an adaptive immune response to it.

Patients with immune deficiency (see Chapter 10) are usually detected clinically by a history of recurrent infection. To determine the competence of the immune system in such patients, a battery of tests is usually conducted (Fig. 2.47); these focus with increasing precision as the nature of the defect is narrowed down to a single element. The presence of the various cell types in blood is determined by routine hematology, often followed by FACS analysis (see Section 2-17) of lymphocyte subsets, and the measurement of serum immunoglobulins. The phagocytic competence of freshly isolated polymorphonuclear leukocytes and monocytes is tested, and the efficiency of the complement system (see Chapter 8) is determined by testing the dilution of serum required for lysis of 50% of antibody-coated red blood cells ($CH_{50}$).

In general, if such tests reveal a defect in one of these broad compartments of immune function, more specialized testing is then needed to determine the precise nature of the defect. Tests of lymphocyte function are often valuable, starting with the ability of polyclonal mitogens to induce T-cell proliferation and B-cell secretion of immunoglobulin in tissue culture (see Section 2-18). These tests eventually pinpoint the cellular defect in immunodeficiency.

**Evaluation of the cellular components of the human immune system**

| | B cells | T cells | Phagocytes |
|---|---|---|---|
| Normal numbers ($\times 10^9$ per liter of blood) | Approximately 0.3 | Total 1.0–2.5<br>CD4 0.5–1.6<br>CD8 0.3–0.9 | Monocytes 0.15–0.6<br>Polymorphonuclear leukocytes<br>Neutrophils 3.00–5.5<br>Eosinophils 0.05–0.25<br>Basophils 0.02 |
| Measurement of function *in vivo* | Serum Ig levels<br>Specific antibody levels | Skin test | — |
| Measurement of function *in vitro* | Induced antibody production in response to pokeweed mitogen | T-cell proliferation in response to phytohemagglutinin or to tetanus toxoid | Phagocytosis<br>Nitro blue tetrazolium uptake<br>Intracellular killing of bacteria |
| Specific defects | See Fig. 10.8 | See Fig. 10.8 | See Fig. 10.8 |

**Evaluation of the humoral components of the human immune system**

| | Immunoglobulins | | | | Complement |
|---|---|---|---|---|---|
| Component | IgG | IgM | IgA | IgE | |
| Normal levels | 600–1400 mg dl$^{-1}$ | 40–345 mg dl$^{-1}$ | 60–380 mg dl$^{-1}$ | 0–200 IU ml$^{-1}$ | $CH_{50}$ of 125–300 IU ml$^{-1}$ |

**Fig. 2.47 The assessment of immunological competence in humans.** Both humoral and cell-mediated aspects of host defense can be checked, usually in a prescribed sequence, to identify the presence of an immune response or the causes of immunological incompetence. The initial screen consists of measuring levels of immunoglobulin and complement, and counting lymphocytes and phagocytic cells. (IgE is present, if at all, at very low levels and is measured in international units (IU) per ml; the $CH_{50}$ of complement is the dilution at which 50% of antibody-coated red blood cells are lysed.) This initial screen usually indicates whether a defect in humoral or T-cell mediated immunity is present, and also whether it affects the induction or mediation of a response. In Chapter 10, defects in host defense known as immunodeficiency diseases are described in detail.

In patients with autoimmune diseases (see Chapter 12), the same parameters are usually analyzed to determine whether there is a gross abnormality in the immune system. However, most patients with such diseases show few abnormalities in general immune function. To determine whether a patient is producing antibody against their own cellular antigens, the most informative test is to react their serum with tissue sections, which are then examined for bound antibody by indirect immunofluorescence using fluorescent-labeled anti-human immunoglobulin (see Section 2-13). Most autoimmune diseases are associated with the production of broadly characteristic patterns of autoantibodies directed at self tissues. These patterns aid in the diagnosis of the disease and help to distinguish autoimmunity from tissue inflammation due to infectious causes.

Related to Part V

## 2-32 Irradiation kills lymphoid cells, allowing the study of immune function by adoptive transfer and the study of lymphocyte development in bone marrow chimeras.

Ionizing radiation from X-ray or γ-ray sources kills lymphoid cells at doses that spare the other tissues of the body. This makes it possible to eliminate immune function in a recipient animal before attempting to restore immune function by adoptive transfer, and allows the effect of the adoptively-transferred cells to be studied in the absence of other lymphoid cells. James Gowans originally used this technique to prove the role of the lymphocyte in immune responses. He showed that all active immune responses could be transferred to irradiated recipients by small lymphocytes from immunized donors. This technique can be refined by transferring only certain lymphocyte subpopulations, such as B cells, CD4 T cells, and so on. Even cloned T-cell lines have been

tested for their ability to transfer immune function, and have been shown to confer adoptive immunity to their specific antigen. Such adoptive transfer studies are a cornerstone in the study of the intact immune system, as they can be carried out rapidly, simply, and in any strain of mouse.

Somewhat higher doses of irradiation eliminate all cells of hematopoietic origin, allowing replacement of the entire hematopoietic system, including lymphocytes, from donor bone marrow stem cells. The resulting animals are called **radiation bone marrow chimeras** from the Greek word *chimera*, a mythical animal that had the head of a lion, the tail of a serpent and the body of a goat. This technique is used to examine the development of lymphocytes as opposed to their effector functioning, and it has been particularly important in studying T-cell development, as we shall see in Chapter 6. Essentially the same technique is used in humans to replace bone marrow when it fails, as in aplastic anemia or after nuclear accidents, or to eradicate the bone marrow and replace it with normal marrow in the treatment of certain cancers.

## 2-33 Genetic defects can prevent the development of all lymphocytes.

There are several inherited immunodeficiencies in humans that are described as severe combined immune deficiency, or SCID, because they are characterized by defects in both humoral and cell-mediated immunity (we shall describe these various syndromes further in Chapter 10). Patients with these disorders suffer from a lack of lymphocytes, or lymphocyte function, and are remarkably susceptible to infection with a wide range of agents: most can survive only if completely isolated from their surroundings. Some SCID patients can be treated by bone marrow transplantation. SCID individuals are a dramatic illustration of the importance of lymphocytes in host defense and of the origin of all lymphocytes from a bone marrow progenitor.

Related to Part V

In the mouse, a recessive mutation called *scid* prevents lymphocyte differentiation (see Chapter 10). Such mice have normal microenvironments for both B- and T-lymphocyte differentiation from stem cells, so grafting normal bone marrow into homozygous *scid/scid* mice can generate an intact immune system. Individual components of the mature immune system can also be transferred to *scid/scid* mice to generate animals expressing only the functions of particular subpopulations of lymphocytes. *Scid* mice are useful for distinguishing those immune functions that are innate (see Chapter 9) as opposed to those that require adaptive immunity mediated by specific lymphocytes. More recent studies use mice mutant in the *RAG-1* and *RAG-2* genes. These mice are completely devoid of functional T and B cells, whereas *scid* mice produce some lymphocytes as they age.

## 2-34 T cells can be eliminated selectively by removal of the thymus or by the *nude* mutation.

The importance of T-cell function *in vivo* can be ascertained in mice with no T cells of their own. Under these conditions, the effect of a lack of T cells can be studied, and T-cell subpopulations can be restored selectively to analyze their specialized functions. T lymphocytes originate in the thymus, and neonatal **thymectomy**, the surgical removal of the thymus of a mouse at birth, prevents T-cell development from occurring because the export of most functionally mature T cells only occurs after birth in the mouse. Alternatively, adult mice can be thymectomized and then irradiated and reconstituted with bone marrow; such mice will develop all hematopoietic cell types except mature T cells.

The recessive *nude* mutation in mice, which in homozygous form causes hairlessness and absence of the thymus, is also associated with failure to develop T cells from bone marrow progenitors. Grafting thymectomized or *nude/nude* mice with thymic epithelial elements depleted of lymphocytes allows the graft recipients to develop normal mature T cells. This procedure allows the role of the non-lymphoid thymic stroma to be examined; it has been crucial in determining the role of thymic stromal cells in T-cell development (see Chapter 6).

## 2-35 B cells are depleted selectively in agammaglobulinemic humans and genetically manipulated mice.

There is no single site of B-cell development in mice, so techniques such as thymectomy cannot be applied to the study of B-cell function and development in rodents. Nor are there mutations equivalent to *nude* that provide one with mice that have T cells but no B cells. However, such mutations exist in humans, leading to a failure to mount humoral immune responses or make antibody. The diseases produced by such mutations are called **agammaglobulinemias**, as they were originally detected as the absence of gamma globulins (see Section 2-9). The genetic basis for one form of this disease in humans has now been established (see Chapter 10), and some features of the disease can be reproduced in mice by targeted disruption of the corresponding gene (see Section 2-37). Several different mutations in crucial regions of immunoglobulin genes have already been produced by gene targeting and have provided mice lacking B cells.

## 2-36 Individual genes can be introduced into mice by transgenesis.

The function of genes has traditionally been studied by observing the effects of spontaneous mutations in whole organisms and, more recently, by analyzing the effects of targeted mutations in cultured cells. The advent of gene cloning and *in vitro* mutagenesis now make possible the analysis of specific mutations within whole animals. Mice with extra copies or altered copies of a gene in their genome can be generated by **transgenesis**, which is now a well established procedure. To produce **transgenic** mice, a cloned gene is introduced into the mouse genome by microinjection into the male pronucleus of a fertilized egg, which is then implanted into the uterus of a pseudopregnant female mouse. In some of the eggs, the injected DNA becomes integrated randomly into the genome, giving rise to a mouse that has an extra genetic element of known structure, the **transgene** (Fig. 2.48). This technique allows one to study the impact of a new gene on development, to identify the regulatory regions of a gene required for its normal tissue specific expression, to determine the effects of its overexpression or expression in inappropriate tissues,

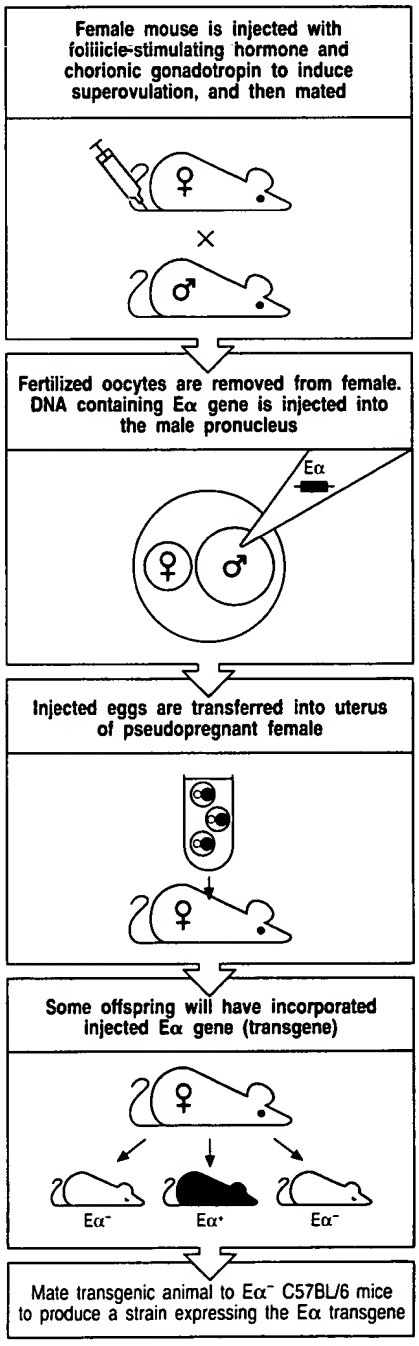


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**Fig. 2.48 The function and expression of genes can be studied *in vivo* using transgenic mice.** DNA encoding a gene of interest, here the mouse MHC class II gene Eα, is purified and microinjected into the male pronucleus of fertilized ova. The ova are then implanted into pseudopregnant female mice. The resulting offspring are screened for the presence of the transgene in their cells, and positive mice are used as founders that transmit the transgene to their offspring, establishing a line of transgenic mice that carry one or more extra genes. The function of the Eα gene used here is tested by breeding the transgene into C57BL/6 mice that carry an inactivating mutation in their endogenous Eα gene.

and to find out the impact of mutations on gene function. Transgenic mice have been particularly useful in studying the role of T-cell and B-cell receptors in lymphocyte development, as will be described in Chapters 5 and 6.

## 2-37 The role of individual genes can be studied *in vivo* by gene knock-out.

In many cases, the functions of a particular gene can only be fully understood if a mutant animal that does not express the gene can be obtained. While genes used to be discovered through identification of mutant phenotypes, it is now more common to discover and isolate the normal gene and then determine its function by replacing it *in vivo* with a defective copy. This procedure is known as **gene knock-out**, and it has been made possible by two fairly recent developments: a powerful strategy to select for targeted mutation by homologous recombination, and the development of continuously growing lines of pluripotent **embryonic stem cells** (**ES cells**).

The technique of **gene targeting** takes advantage of the phenomenon known as **homologous recombination** (Fig. 2.49). Cloned copies of the target gene are altered to make them non-functional and are then introduced into the ES cell where they recombine with the homologous gene in the cell's genome, replacing the normal gene with a non-functional copy. Homologous recombination is a rare event in mammalian cells,

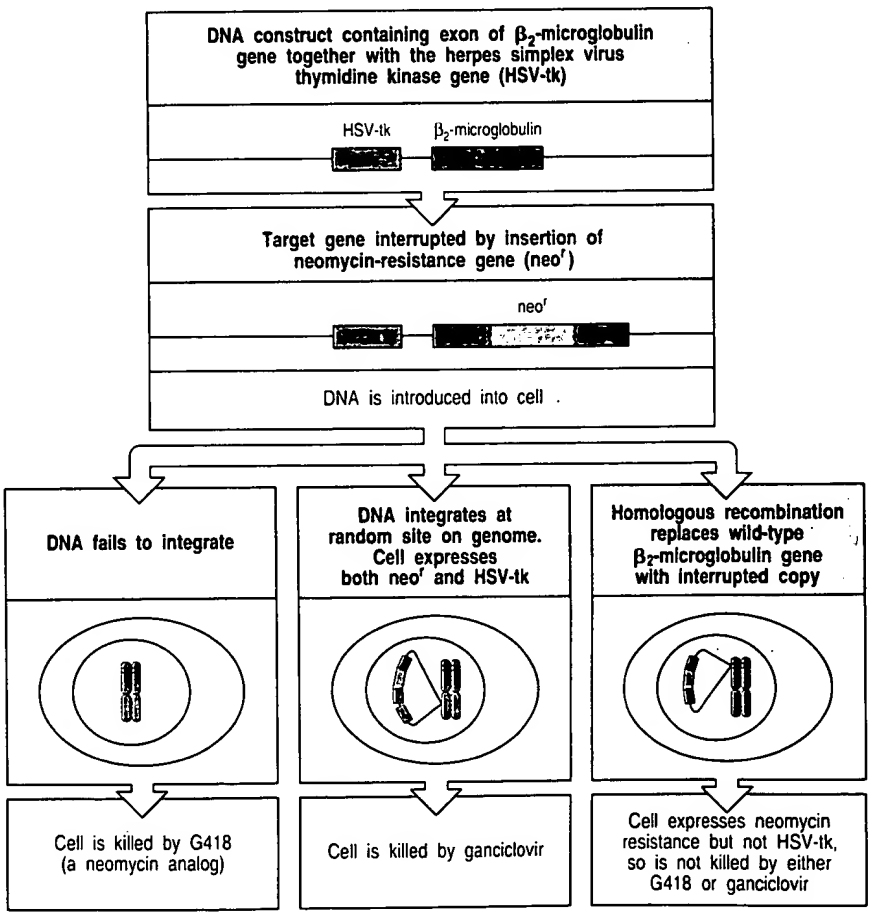


**Fig. 2.49 The deletion of specific genes can be accomplished by homologous recombination.** When pieces of DNA are introduced into cells, they can integrate into cellular DNA in two different ways. If they randomly insert into sites of DNA breaks, the whole piece is usually integrated, often in several copies. However, extrachromosomal DNA can also undergo homologous recombination with the cellular copy of the gene, in which case only the central, homologous region is incorporated into cellular DNA. Inserting a selectable marker gene such as resistance to neomycin ($neo^r$) into the coding region of a gene does not prevent homologous recombination, and it achieves two goals. First, it protects any cell that has integrated the injected DNA from the neomycin-like antibiotic G418. Second, when the gene recombines with homologous cellular DNA, the $neo^r$ gene disrupts the coding sequence of the modified cellular gene. Homologous recombinants can be discriminated from random insertions if the gene for herpes simplex virus thymidine kinase (HSV-tk) is placed at one or both ends of the DNA construct, which is often known as a 'targeting construct' because it targets the cellular gene. In random DNA integrations, HSV-tk is retained. HSV-tk renders the cell sensitive to the anti-viral agent ganciclovir. However, as HSV-tk is not homologous to the target DNA, it is lost from homologous integrants. Thus, cells that have undergone homologous recombination are uniquely both $neo^r$- and ganciclovir-resistant, and survive in a mixture of the two antibiotics. The presence of the disrupted gene has to be confirmed by Southern blotting or by the polymerase chain reaction (PCR) using primers in the $neo^r$ gene and in cellular DNA lying outside the region used in the targeting construct. By using two different resistance genes one can disrupt the two cellular copies of a gene, making a deletion mutant (not shown).

Related to Part V

and thus a powerful selection strategy is required to detect those cells in which it has occurred. Most commonly, the introduced gene construct has its sequence disrupted by an inserted antibiotic-resistance gene such as that for neomycin resistance. If this construct undergoes homologous recombination with the endogenous copy of the gene, the endogenous gene is disrupted but the antibiotic-resistance gene remains functional, allowing cells that have incorporated the gene to be selected in culture for resistance to the neomycin-like drug G418. Antibiotic resistance on its own shows only that the cells have taken up and integrated the neomycin-resistance gene. To select for those cells in which homologous recombination has occurred, the ends of the construct usually carry the thymidine kinase gene from the herpes simplex virus (HSV-tk). Cells that incorporate DNA randomly usually retain the entire DNA construct including HSV-tk, whereas homologous recombination between the construct and cellular DNA, the desired result, involves the exchange of homologous DNA sequences so that the non-homologous HSV-tk genes at the ends of the construct are eliminated. Cells carrying HSV-tk become sensitive to the anti-viral drug, ganciclovir, and so cells with homologous recombinations have the unique feature of being resistant to both neomycin and ganciclovir, allowing them to be selected efficiently when these drugs are added to the cultures (see Fig. 2.49).

This technique can be used to produce homozygous mutant cells in which the effects of knocking-out a specific gene can be analyzed. Diploid cells in which both copies of a gene have been mutated by homologous recombination can be selected after transfection with a mixture of constructs in which the gene to be targeted has been disrupted by one or other of two different antibiotic resistance genes. Having obtained a mutant cell with a functional defect, the defect can be ascribed definitively to the mutated gene if the mutant phenotype can be reverted with a copy of the wild-type gene transfected into the mutant cell. Restoration of function means that the defect in the mutant gene has been complemented by the wild-type gene's function. This technique is very powerful, since it allows the gene that is being transferred to be mutated in precise ways to determine which parts of the protein are required for function.

Related to Part V

To knock out a gene *in vivo*, it is only necessary to disrupt one copy of the cellular gene in an embryonic stem cell. Embryonic stem cells carrying the mutant gene are produced by targeted mutation (as in Fig. 2.49), and injected into a blastocyst which is re-implanted into the uterus. The mutated cells become incorporated into the developing embryo and contribute to all tissues of the resulting chimeric offspring including the germline. The mutated gene can therefore be transmitted to some of the offspring of the original chimera, and further breeding of the mutant gene to homozygosity produces mice that completely lack the expression of that particular gene product (Fig. 2.50). The effects of the absence of the gene's function can then be studied. In addition, the parts of the gene that are essential for its function can be identified by determining whether function can be restored by introducing different mutated copies of the gene back into the genome by transgenesis. The manipulation of the mouse genome by gene knock-out and transgenesis is revolutionizing our understanding of the role of individual genes in lymphocyte development and function, as we shall see throughout this book.

A problem with gene knock-outs arises when the function of the gene is essential for the survival of the animal; in such cases the gene is termed a **recessive lethal gene** and homozygous animals cannot be produced. However, by making chimeras with mice that are deficient in B and T cells, it is possible to analyze the function of recessive lethal genes in lymphoid cells. To do this, ES cells with homozygous lethal loss-of-function mutations are injected into blastocysts of mice lacking the ability to

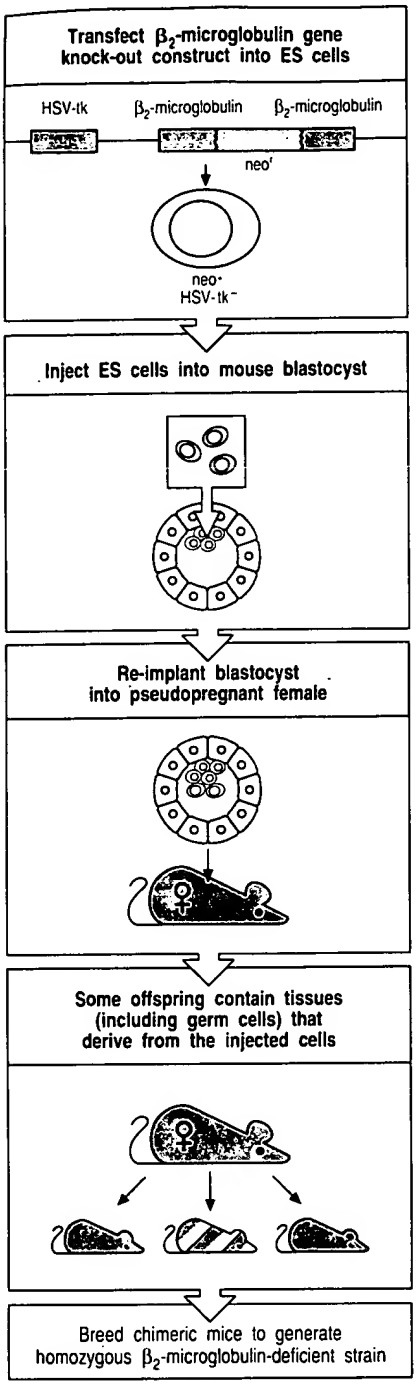


**Fig. 2.50 Gene knock- ut in mbry nic stem (ES) cells enables mutant mice to be pr duced.** Specific genes can be inactivated by homologous recombination in tissue cultures of cells known as embryonic stem cells, which, on implantation into a blastocyst, can give rise to all cell lineages in a chimeric mouse. The technique of homologous recombination is carried out as described in Fig. 2.48. In this example, the gene for $\beta_2$-microglobulin is disrupted by homologous recombination of a targeting construct in ES cells. Only a single copy of the gene needs to be disrupted. ES cells in which homologous recombination has taken place are injected into mouse blastocysts. If the mutant ES cells give rise to germ cells in the resulting chimeric mice (striped in the figure), then the mutant gene can be transferred to their offspring. By breeding the mutant gene to homozygosity, a mutant phenotype is generated. In this case, the homozygous mutant mice lack MHC class I molecules on their cells, as MHC class I molecules have to pair with $\beta_2$-microglobulin for surface expression. The $\beta_2$-microglobulin-deficient mice can then be bred with mice transgenic for subtler mutants of the deleted gene, allowing the effect of such mutants to be tested *in vivo*.

rearrange their antigen receptor genes because of a mutation in their recombinase-activating genes (*RAG* knock-out mice). As these chimeric embryos develop, the *RAG*-deficient cells can compensate for any developmental failure resulting from the gene knock-out in the ES cells, in all but the lymphoid lineage. So long as the mutated ES cells can develop into hematopoietic progenitors in the bone marrow, the embryos will survive, and all the lymphocytes in the resulting chimeric mouse will be derived from the mutant ES cells (Fig. 2.51).

A second, powerful technique achieves tissue-specific or developmentally regulated gene deletion by employing the DNA sequences and enzymes used by bacteriophage P1 to excise itself from a host cell's genome. The integrated phage DNA is flanked by recombination signal sequences, called *loxP* sites. A recombinase, Cre, recognizes these sites, cuts the DNA and joins the two ends, thus excising the intervening DNA in the form of a circle. This mechanism can be adapted to allow the deletion of specific genes in a transgenic animal only in certain tissues or at certain times in development. First, *loxP* sites flanking a gene, or perhaps just a single exon, are introduced by homologous recombination (Fig. 2.52). Usually, the introduction of these sequences into flanking or intronic DNA does not disrupt the normal function of the gene. Mice containing such *loxP* mutant genes are then mated with mice made transgenic for the Cre recombinase, under the control of a tissue-specific or inducible promoter. When the Cre recombinase is active, either in the appropriate tissue or when induced, it excises the DNA between the inserted *loxP* sites, thus inactivating the gene or exon. Thus, using a T-cell specific promoter to drive expression of the Cre recombinase, a gene can be deleted only in T cells, while remaining functional in all other cells of the animal. This is an extremely powerful genetic technique that is still in its infancy and is certain to yield exciting results in the future.

Related to Part V

## Summary.

The measurement of immune function in intact organisms is essential to a full understanding of the immune system in health and disease. The ability of an immunized individual to resist infection is still the standard assay for protective immunity conferred by infection or vaccination. Local reactions to antigens injected into the skin can provide information

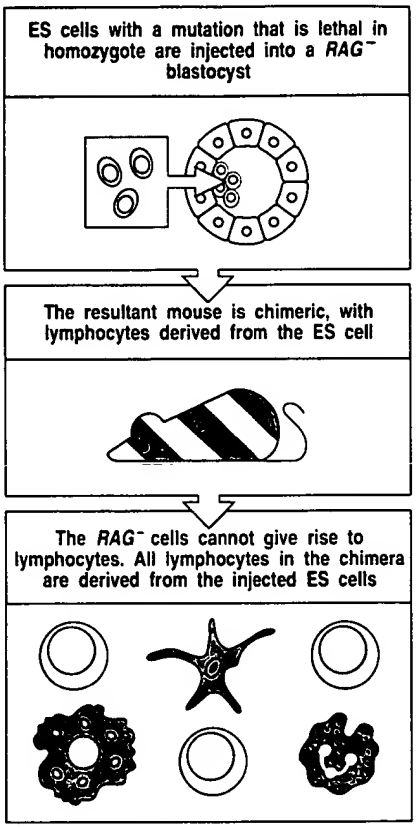


**Fig. 2.51 The role of recessive lethal genes in lymphocyte functi n can be studied using *RAG*-deficient chimeric mice.** Embryonic stem (ES) cells carrying the lethal mutation are injected into a *RAG*-deficient blastocyst (top panel). The *RAG*-deficient cells can give rise to all the tissues of a normal mouse except lymphocytes, and so can compensate for any deficiency in the developmental potential of the mutant ES cells (middle panel). If the mutant ES cells are capable of differentiating into hematopoietic stem cells, that is, if the gene function that has been deleted is not essential for this developmental pathway, then all the lymphocytes in the chimeric mouse will be derived from the ES cells (bottom panel), as *RAG*-deficient mice cannot make lymphocytes of their own.

about antibody and T-cell responses to the antigen, a procedure that is particularly important in testing for allergic reactions. Finally, many *in vitro* assays such as the analysis of specific antibody in serum and the proliferative responses of T cells to mitogens and specific antigen are used to assess immune function in human patients.

Manipulation of the immune system *in vivo* reveals the need for each of its components. Using irradiation or mutation to eliminate lymphocytes or particular lymphocyte lineages, and then adoptively transferring mature lymphocytes, isolated subpopulations, cloned T-cell lines, or bone marrow stem cells allows one to study the functions and development of individual normal or immune cell types in an *in vivo* setting. The role of individual genes in lymphocyte development and function can be studied *in vivo* by manipulating the mouse genome, adding genes by transgenesis or eliminating them through gene knock-out. These two techniques can be combined to give detailed information about structure–function relationships in genes and their protein products, either in cultured cells or *in vivo*. These powerful techniques are increasing our understanding of immunobiology at an astonishing rate. The use of mutant mice in the study of host defenses to specific pathogens should provide a new understanding of these highly complex processes.

Related to Part V

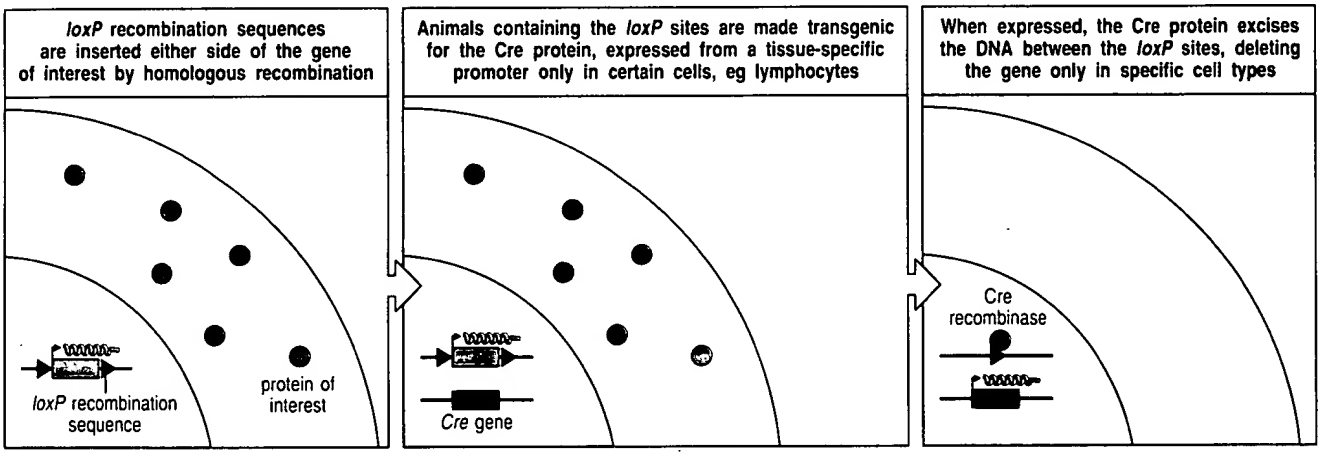


**Fig. 2.52 The P1 bacteriophage recombination system can be used to eliminate genes in particular cell lineages.** The P1 bacteriophage protein Cre will excise DNA that is bounded by recombination signal sequences called *loxP* sequences. These sequences can be introduced at either end of a gene by homologous recombination (left panel). Animals carrying *loxP*-flanked genes can also be made transgenic for the gene for the Cre protein, which is placed under the control of a tissue-specific promoter so that it is only expressed in certain cells or at certain times during development (middle panel). In the cells in which the Cre protein is expressed, it recognises the *loxP* sequences and excises the DNA lying between them (right panel). Thus, individual genes cen be deleted only in certain cell types or only at certain times. In this way, genes that are essential for the normal development of a mouse may be deleted from, for example, T cells, and the role of such genes in T-cell function studied. Genes are shown as boxes, RNA as squiggles, and proteins as coloured balls.

## Summary to Chapter 2.

The immune system is very complex. To analyze it properly, it must be broken down into its individual components, and these must be studied both in isolation and in the context of the larger system. In this chapter we have described how immune responses are induced and measured, and how the immune system can be manipulated experimentally. An appreciation of the methodologies and findings described in this chapter is essential for a full understanding of immunobiology, and many of the techniques included here are used routinely in the experiments described in subsequent chapters. Some, especially the use of monoclonal antibodies for identifying molecules in cells and tissues and the manipulation of the mouse genome, also have general applications in biology.

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